Factors affecting the selectivity of composts suitable for the cultivation of Agaricus species.

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FACTORS AFFECTING THE SELECTIVITY OF COMPOSTS SUITABLE FOR THE CULTIVATION OF AGARICUS SPECIES

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ABSTRACT

Mycelial growth rates of a number of *Agaricus* spp. were compared in a range of traditionally prepared mushroom composts that had received extended composting periods at 45°C and 55°C. It became evident that mycelial growth rate in composted substrates can be suppressed by NH_4^+ levels within the compost above 500 ppm. Although a fast colonisation of the substrate by mushroom mycelium is highly beneficial for excluding fungal contaminants, an improvement in yield is not necessarily guaranteed.

The nutritional status of prepared composts was assessed, using a sonication technique with dilute alkali, to extract from freeze dried compost samples, four distinct fractions; debris 1 (the 'cleaned' cereal straw fraction), debris 2 (a microbial fraction embedded in humic material), humic acid (an alkali soluble/acid insoluble fraction), and a clear supernatant (fulvic acid). Depending on the duration of composting, the humic and debris 2 fractions represented between 12-15% and 15-30% of the compost dry matter respectively and collectively the nitrogen bound in both fractions amounted to approximately 40% of the compost nitrogen.

Using a model growing system (deep columns of compost), the disappearance of these compost fractions in relation to extracellular enzyme production (laccase, endocellulase, β-N-acetyl glucosaminidase, β-N-acetyl muramidase) by the mushroom was studied. The debris 2 fraction was shown to decline markedly once the substrate had become totally colonised by mushroom mycelium and there would seem to be a direct relationship between the disappearance of this fraction and the peak in laccase activity.

There was also evidence that the protein content of the humic fraction decreased during the cropping period although the rate of disappearance, unlike the debris 2 fraction, appeared uniform with depth of substrate. It is clear from the work presented in this thesis that during mycelial colonisation and fructification there are zones of differential activity operating within the substrate.

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LIST OF CONTENTS

		Page
Title Page		1
Abstract		2
Acknowledgements		4
List of Contents		5
Appendices		9
List of Tables		10
List of Figures		11
List of Plates		14
CHAPTER 1 : INTRODUCTION		16
1.1	Mushroom Production	16
1.2	Evolution of mushroom composting technology	17
1.3	The role of micro-organisms in compost	24
1.4	Compost Selectivity or Specificity	27
1.5	The humus fraction of mushroom composts	34
1.6	Extracellular enzyme activities of *Agaricus bisporus* in composted substrates	37
1.7	Mushroom mycelium growth rate as an indicator of compost selectivity	41
1.8	Concluding comments on compost selectivity	42
CHAPTER 2 : MATERIALS AND METHODS		44
2.1	Compost materials	44
2.2	*Agaricus* species	44
2.3	Design of bench-scale composting units	45
2.4	Substrate preparation and modification	46
2.5	Mushroom inoculum (spawn) preparation	47

2.6	Methods of compost analysis	47
	2.6.1 Moisture, pH and total ash determination	47
	2.6.2 Total Nitrogen	49
	2.6.3 Ammonia	49
	2.6.4 Ammonium Nitrogen	50
	2.6.5 Soluble carbohydrate	50
2.7	Measurement of fungal growth in compost - 'Race Tube' technique	51
2.8	Measurement of fungal growth in liquid culture	51
2.9	Extraction and analysis of humic substances	52
	2.9.1 Sonication technique	52
	2.9.2 Analysis of humic and fulvic fractions	53
	(1) Soluble carbohydrate (2) Phenol content (3) Total protein	
2.10	Assembly of compartmentalised deep column	54
2.11	Measurement of laccase activity	55
2.12	Measurement of extracellular endocellulase	56
2.13	Measurement of β-N-acetyl muramidase	57
2.14	Measurement of β-N-acetylglucosaminidase	57
2.15	Determination of proteolytic activity	58
2.16	Statistical Analysis	58

CHAPTER 3 : MYCELIAL COLONISATION RATE OF COMPOSTED SUBSTRATES AS A MEASUREMENT OF COMPOST SELECTIVITY

3.1	Procedure	60
3.2	Experiments 1 & 2 (Prolonged composting at 45°C)	60
3.3	Experiment 3 (Prolonged composting at 45°C & 55°C)	68
3.4	Experiments 4 & 5 (Prolonged composting at 45°C & 55°C with/without glucose supplementation)	69
3.5	Experiments 6 & 7 (Confirmation expt., no carbohydrate supplementation)	75

3.6 Experiment 8 (Prolonged composting of H.R.I. compost at 45°C & 55°C; laccase and ammonium ion determinations) 81

3.7 Experiment 9 (Prolonged composting of Blue Prince compost at 45°C & 55°C) 89

3.8 Experiment 10 (Prolonged composting of H.R.I. compost at 45°C and its effect on mushroom yield) 89

3.9 Mycelial growth on 2% malt agar supplemented with increasing concentrations of ammonium salts 94

3.10 The effect of increasing ammonium ion concentration on fungal biomass and laccase activity in liquid culture 94

3.11 Concluding comments 100

CHAPTER 4 : ASSESSMENT OF HUMIC MATERIALS THAT ACCUMULATE DURING THE COMPOSTING PROCESS AND THEIR ROLE IN MUSHROOM NUTRITION

4.1 Development of a reliable extraction procedure to assess the alkali soluble/alkali insoluble fraction (humic acid) of composted substrates 104

4.2 Sonication time experiments S1-S4 105

4.3 Changes in the proportion of debris 1, debris 2, humic and fulvic fractions during the prolonged composting of two distinct compost types (Expts. 8 & 9) 110

4.4 Analyses of 6 Controlled Environment Composts (C.E.C.) with increasing N contents and assessment of humic, fulvic, debris 1 and debris 2 fractions in relation to compost productivity 120

4.5 Nitrogen balance in debris 1, debris 2, humic and fulvic fractions in a high and low N compost formulation at the commencement and completion of composting 121

4.6 Concluding Comments 125

CHAPTER 5 : ASSESSMENT OF DEBRIS 1, DEBRIS 2, HUMIC AND FULVIC ACID CHANGES IN COMPOSTS DURING MYCELIAL COLONISATION AND MUSHROOM PRODUCTION.

5.1 Small scale investigation using 'race tubes' 135

5.2 Comparison of enzyme profiles (laccase, endo-cellulase, β-N-acetyl glucosaminidase and β-N-acetyl muramidase) with substrate depth and association with changes in compost constituents 136

5.3 Changes in protein, carbohydrate and phenolic content of ash-free humic acid in compost samples isolated from cased and uncased compost columns 145

5.4 Concluding comments 146

CHAPTER 6 GROWTH OF MUSHROOM MYCELIUM ON SOLID AND LIQUID MEDIA

6.1 Mycelial growth on water agar supplemented with humic or debris 2 fractions as the sole nutrient source 161

6.2 Mycelial growth on minimal glucose media supplemented with either humic or debris 2 fractions as the sole nitrogen source 162

6.3 Induction of proteinase by mushroom mycelium when grown in defined media (Treschow's) using humic fraction as the sole nitrogen source 162

6.4 Concluding comments 170

CHAPTER 7 DISCUSSION 172

7.1 Mycelial growth in compost and how it relates to compost selectivity 172

7.2 Humic material and its role in compost selectivity and mushroom nutrition 176

7.3 Sequential degradation of compost fractions by mushroom enzymes 181
7.4 Concluding Comments 184

REFERENCES 187

APPENDICES

Page

1. Liquid Media (based on Treschow, 1944) 197
2. Analytical data for Experiment 8 (H.R.I.) composts (45°C) 198
3. Analytical data for Experiment 8 (H.R.I.) composts (55°C) 199
4. Protein/Carbohydrate/Phenolic content (%) of Ash-free Humic Fraction of Experiment 8 (H.R.I.) composts 200
5. Protein/Carbohydrate/Phenolic content (%) of Fulvic Acid Fraction of Experiment 8 (H.R.I.) composts 201
6. Analytical data for Experiment 9 (Blue Prince) composts (45°C) 202
7. Analytical data for Experiment 9 (Blue Prince) composts (55°C) 203
8. Protein/Carbohydrate/Phenolic content (%) of Ash-free Humic Fraction of Experiment 9 (Blue Prince) composts 204
9. Ammonium ion concentrations (% dry compost matter) present in Compost Expts. 8, 9 & 10. 205
10. Protein/Carbohydrate/Phenolic content (%) of Fulvic Acid Fraction of Experiment 9 (Blue Prince) composts 206
11. Yield of mushrooms (g) and Production Efficiencies (P.E.) obtained from 4 compost types (Experiment 10) 207
12. Estimation of Mushroom Biomass during colonisation of compost in Race Tubes (See 5.1) 208
13. Ash content of Debris 1 and Debris 2 Fractions 208
14. Moisture content of 5 separate compost layers within the cased and uncased columns (See Chapter 5) 209

LIST OF TABLES

		Page
Table 1.	Major groups and succession of micro-organisms during compost preparation	26
Table 2.	Extracellular enzyme activities of *Agaricus bisporus* .	40
Table 3.	Changes of composition in a traditionally prepared mushroom compost when subjected to further composting periods of 1, 2 and 3 weeks at 45°C	62
Table 4.	Mycelial advance (mm/day in 'race tubes') of 7 *Agaricus* strains in a traditionally prepared compost that had received 1, 2 and 3 weeks prolonged composting at 45°C prior to inoculation	63
Table 5.	Analysis of changes occurring in a traditionally prepared mushroom compost when subjected to further composting periods of 1, 2 and 3 weeks at 45°C and 55°C	70
Table 6.	Mycelial advance (mm/day in 'race tubes') of 6 *Agaricus* strains in a traditionally prepared compost that had received 1, 2 and 3 weeks prolonged composting at 45°C and 55°C prior to inoculation	71
Table 7.	Analysis of changes occurring within composts during prolonged composting periods at 45°C with/without glucose supplementation (Experiment 4)	72
Table 8.	Analysis of changes occurring within composts during prolonged composting periods at 55°C with/without glucose supplementation (Experiment 5)	72
Table 9.	Analysis of changes occurring within composts during prolonged composting periods at 45°C (Expt.6) and 55°C (Expt.7) with/without glucose supplementation	76
Table 10.	Mycelial advance (mm/day in 'race tubes') of *Agaricus* spp. in a traditionally prepared compost that had received 1, 2 and 3 weeks prolonged composting at 45°C (Expt.6) and 55°C (Expt.7) prior to inoculation	77
Table 11.	Laccase activity of 6 *Agaricus* spp. within colonised compost (μ moles O_2/g dry matter) assayed 21 days after mycelial inoculation	78
Table 12.	Analytical changes occurring within composts during prolonged composting periods at 45°C and 55°C (Experiment 8)	82
Table 13.	Analytical changes occurring within composts during prolonged composting periods at 45°C tand 55°C and its effect on mushroom growth (Experiment 9)	90
Table 14.	Analytical changes occurring within composts during prolonged composting periods at 45°C and its effect on mushroom growth (Experiment 10)	91

Page

Table 15. Nitrogen Balance of Debris 1, Debris 2, Humic and Fulvic fractions in a high nitrogen compost formulation at the commencement of composting (3 separate analyses combined) 123

Table 16. Nitrogen Balance of Debris 1, Debris 2, Humic and Fulvic fractions in a high nitrogen compost formulation at the completion of Phase 2 composting (3 separate analyses combined) 123

Table 17. Nitrogen Balance of Debris 1, Debris 2, Humic and Fulvic fractions in a low nitrogen compost formulation at the commencement of composting (2 separate analyses combined) 124

Table 18. Nitrogen Balance of Debris 1, Debris 2, Humic and Fulvic fractions in a low nitrogen compost formulation at the completion of Phase 2 composting (2 separate analyses combined) 124

Table 19. Analyses of Controlled Environment Composts (1-6) at the start and completion of composting 131

LIST OF FIGURES

Figure 1. Chemical changes occurring in mushroom substrates during the composting cycle 38

Figure 2. Multi-adaptor flask fermentation unit 48

Figure 3. Flowchart of extraction of humic and fulvic acids from mushroom compost 59

Figure 4. Race-tube mycelial growth rates of *Agaricus* spp. (Expt. 1) 64

Figure 5. Race-tube mycelial growth rates of *Agaricus* spp. (Expt. 1) 65

Figure 6. Race-tube mycelial growth rates of *Agaricus* spp. (Expt. 2) 66

Figure 7. Race-tube mycelial growth rates of *Agaricus* spp. (Expt. 2) 67

Figure 8. Soluble carbohydrate analyses of compost that had received prolonged composting times of 1, 2 and 3 weeks at 45°C and 55°C (Expts. 4 & 5) 73

Figure 9. Comparison of mycelial growth rates in composts receiving additional composting times of 1, 2 and 3 weeks at 45°C and 55°C (Expts. 4 & 5) 74

Figure 10. Comparisons of rate of mycelial colonisation in compost with laccase activity and ammonium ion concentration (prolonged composting at 45°C; Expt. 8) 84

Page

Figure 11.	Comparisons of rate of mycelial colonisation in compost with laccase activity and ammonium ion concentration (prolonged composting at 55°C; Expt. 8)	85
Figure 12.	Comparisons of rate of mycelial colonisation in compost with laccase activity and ammonium ion concentration (prolonged composting at 45°C - autoclaved series; Expt. 8)	86
Figure 13.	Comparisons of rate of mycelial colonisation in compost with laccase activity and ammonium ion concentration (prolonged composting at 55°C - autoclaved series; Expt. 8)	87
Figure 14.	Comparisons of rate of mycelial colonisation in compost with laccase activity and ammonium ion concentration (prolonged composting at 45°C; Expt. 10)	92
Figure 15.	Fresh weight of mushrooms harvested per 100g dry matter (Production Efficiency) from 4 compost types	96
Figure 16.	Mycelial growth on 2% malt agar supplemented with ammonium salts	97
Figure 17.	The effect of increasing NH_4^+ ion on fungal biomass and laccase activity in liquid culture	99
Figure 18.	The effect of increasing sonication time on the estimation of Humic, Debris 1 and Debris 2 fractions of freeze dried H.R.I. compost samples (Expts. S1 & S2)	106
Figure 19.	The effect of increasing sonication time on the estimation of Humic, Debris 1 and Debris 2 fractions of freeze dried H.R.I. compost samples (Expts. S3 & S4)	107
Figure 20.	Analysis of Soluble Carbohydrates in the Fulvic Fraction	108
Figure 21.	Effect of prolonged composting times on the proportions of Debris 1, Debris 2, Humic and Fulvic fractions of compost (Expt. 8)	111
Figure 22.	Protein/Carbohydrate/Phenolic content of Ash-Free Humic Material (Expt. 8)	112
Figure 23.	Protein/Carbohydrate/Phenolic content of Humic Fraction (Expt. 8) (as a %age of ash-free compost dry matter)	113
Figure 24.	Protein/Carbohydrate/Phenolic content of Fulvic Fraction (Expt. 8) (as a %age of compost dry matter)	114
Figure 25.	Effect of prolonged composting times on the proportions of Debris 1, Debris 2, Humic and Fulvic fractions of composts (Expt. 9)	115
Figure 26.	Protein/Carbohydrate/Phenolic content of Ash-Free Humic Material (Expt. 9)	116
Figure 27.	Protein/Carbohydrate/Phenolic content of Humic Fraction (Expt. 9) (as a %age of ash-free compost dry matter)	117
Figure 28.	Protein/Carbohydrate/Phenolic content of Fulvic Fraction (Expt. 9) (as a %age of compost dry matter)	118

		Page
Figure 29.	Ash-Free Humic content of two distinct compost types	127
Figure 30.	Changes in Debris 2 content (% compost dry matter) of two distinct compost types during extended composting periods	128
Figure 31.	Changes in Debris 2 content (% ash-free compost dry matter) of two distinct compost types during extended composting periods	129
Figure 32.	Compost Components of 6 "Controlled Environment" Composts	131
Figure 33.	Changes in the Debris 2 and Humic Fractions of compost in relation to Laccase activity	137
Figure 34.	Changes in the proportions of compost fractions during composting and throughout mycelial colonisation (% compost dry matter)	138
Figure 35.	Changes in the proportions of compost fractions during composting and throughout mycelial colonisation (% compost dry matter; corrected for constant ash)	139
Figure 36.	Extracellular endocellulase activity within 5 layers of the cased compost column	147
Figure 37.	Laccase assays on cased and uncased compost columns	148
Figure 38.	*β*-N-acetyl glucosaminidase activity in cased and uncased compost columns	149
Figure 39.	*β*-N-acetyl muramidase activity in cased and uncased compost columns	150
Figure 40.	Utilisation of Debris 1 by mushroom mycelium within 5 separate compost layers of the deep column	151
Figure 41.	Utilisation of Debris 1 by mushroom mycelium within 5 separate compost layers of the deep column (corrected for constant ash)	152
Figure 42.	Assessment of Debris 2 remaining within 5 separate compost layers of the deep column	153
Figure 43.	Assessment of Debris 2 remaining within 5 separate compost layers of the deep column (corrected for constant ash)	154
Figure 44.	Assessment of Ash-Free Humic material within 5 separate compost layers of the deep column (corrected for constant ash)	155
Figure 45.	Protein content of Ash-Free Humic material of compost sampled from top, middle and bottom layers of cased and uncased compost columns	156
Figure 46.	Carbohydrate content of Ash-Free Humic material of compost sampled from top, middle and bottom layers of cased and uncased compost columns	157

		Page
Figure 47.	Phenolic content of Ash-Free Humic material of compost sampled from top, middle and bottom layers of cased and uncased compost columns	158
Figure 48.	Growth of Mushroom Mycelium on Water Agar supplemented with Humic and Debris 2 fractions	164
Figure 49.	Growth of Mushroom Mycelium on Minimal Media supplemented with Humic Material	165
Figure 50.	Growth of Mushroom Mycelium on Minimal Media supplemented with Debris 2	166
Figure 51.	Growth of Mushroom Mycelium on Minimal Media supplemented with Humic/Debris 2 mixtures	167
Figure 52.	Fungal Biomass accumulation in Treschow's Medium when Glutamate (sole N source) is replaced by Humic Acid and Casein	168
Figure 53.	Induction of Proteinase in Treschow's Media when Glutamate (sole N source) is replaced by Humic Acid and Casein	169

LIST OF PLATES

Plate 1.	Comparison of mycelial growth of 6 *Agaricus* spp. after 21 days in composts that had received prolonged composting times at 45°C for 1, 2 and 3 weeks (Experiment 6)	79
Plate 2.	Comparison of mycelial growth of 6 *Agaricus* spp. after 21 days in composts that had received prolonged composting times at 55°C for 1, 2 and 3 weeks (Experiment 7)	80
Plate 3.	Comparison of mycelial growth of *Agaricus bisporus* (U3) in sterile and unsterile composts that had received prolonged composting times at 45°C and 55°C for 1 week (Expt. 8)	83
Plate 4.	Effect of increasing ammonium ion concentrations (ammonium tartrate) on radial growth of *Agaricus bisporus* (U3) on agar	95
Plate 5.	Effect of increasing ammonium ion concentrations (ammonium tartrate) on fungal biomass (*A. bisporus;* U3) in liquid culture	98
Plate 6.	Freeze dried compost sample before sonication (Bar=5μm)	132
Plate 7.	Freeze dried compost sample after 10 minutes sonication with 0.5M NaOH (Bar = 5μm)	132
Plate 8.	Debris 1 fraction showing straw fragments with humic layer removed (Bar = 100μm)	133

		Page
Plate 9.	Alkali insoluble debris 2 separated by centrifugation at 9,000 rpm (Bar = 5μm)	133
Plate 10.	Intact microbial colonies separating from vascular bundle during alkali extraction (Bar = 10μm)	134
Plate 11.	Cross sectional view of cereal straw vascular system after extraction of humic and microbial biomass (Bar= 10μm)	134
Plate 12.	Compost column (1.5m high) divided into 5 separate compartments (top, upper middle, middle, lower middle and bottom) to facilitate compost sampling	140
Plate 13.	Compost column with side panels removed to show compost sampling points. N.b. Compost sampled initially on left hand side; further samples taken to the right	141
Plate 14.	Debris 2 fraction isolated from fresh weight compost sample (x200). N.b. high proportion of bacterial cells and fungal fragments	163

CHAPTER 1 INTRODUCTION

1.1 Mushroom Production

Mushroom science can be regarded as still in its infancy, although the upsurge in mushroom research over the last thirty years, into compost chemistry, microbiology, mushroom breeding techniques and crop management has led to a threefold increase in mushroom production. In 1992, over 118,000 tonnes of mushrooms were produced (MAFF, 1993) in the UK with a retail crop value in excess of £250 million, making this crop the most valuable of all the protected crops.

Today, the process of producing a selective medium for *Agaricus bisporus* from wheat straw plus animal manures, has evolved into a highly sophisticated commercial operation incorporating two distinct composting phases or stages. Phase 1 is performed as windrow stacks, normally 2m x 2m cross section, and generally protected from the elements by a covered barn. Depending on the physical condition of the starting ingredients, Phase 1 composting is normally completed within 8-10 days, during which time the materials are mixed every 2-3 days and restacked using a turning machine. It is not uncommon for stack temperatures to reach 80°C during this initial phase. Phase 2 composting commences after the compost is filled into shelves, trays or, as on most modern farms today, into long rectangular-shaped buildings normally referred to as tunnels, where it is initially subjected to a pasteurisation temperature around 58-60°C (Fermor et al, 1985). This is usually achieved by raising the air temperature with steam. While a pasteurisation period of at least 3 hours is sufficient to kill insect pests, fungal pathogens and viruses, resident populations of thermotolerant and thermophilic microorganisms remain unaffected. On completion of the pasteurisation phase, fresh filtered air is introduced into the facility to reduce the compost temperature to around 45-50°C. This temperature, which is more favourable for development of thermotolerant and thermophilic microbial

populations, is maintained for a further 3-4 days until the compost is free of ammonia and sweet smelling. Phase 2 composting is normally completed within 7-10 days and mushroom spawn is applied when the compost temperature has fallen to around 25°C.

Although the composting technology used today on the majority of farms produces a selective medium on which to culture the cultivated mushroom *Agaricus bisporus*, there is still much to be learned about the microbial populations within the compost and the effect that they have on the eventual chemical and biochemical constitution of the resulting substrate. The accepted compost technology of today has resulted from empirical approaches to the subject over many decades, but now a more fundamental study of compost selectivity and productivity is needed if composting technology is to be further improved.

1.2 Evolution of mushroom composting technology

Abercrombie (1779) was possibly the first worker to identify the essential procedures associated with the production of a selective medium for the cultivation of the mushroom *Agaricus bisporus*. He recognised that mixtures of raw materials, such as cereal straws and animal manures required wetting, stacking and periodic mixing if a suitable composted substrate on which to cultivate *A. bisporus* was to be achieved. His method of composting stable manure in stacks up to 50ft long, 4-5ft wide and 4-5ft high was the first published method to bear a resemblance to the procedure used today. This simple single phase composting technique, which could last 6 weeks or more before compost temperatures subsided, fitted in well with the outdoor ridge bed technique of mushroom growing. In this system there was virtually no movement of the composted materials on the farm, other than a periodic turning of the compost *in-situ* (Smith, 1993). Cool, compacted beds were then inoculated with manure colonised with mushroom mycelium (manure spawn) and after a period of colonisation were covered with a loam soil to induce fruit-body production. Depending on the weather conditions,

mushrooms were produced from such beds during the Spring and Autumn months.

Callow (1831) observed that compost variability was closely related to the environmental conditions prevailing during compost preparation and was probably the first worker to suggest that a better control of composting could be achieved when the stacks were protected from the elements. He also suggested that a more controlled environment for mushroom production e.g. purpose built, shelved growing houses, would be advantageous for crop management and all the year round growing. Nevertheless, the outdoor method of production remained popular for many decades, possibly for economic reasons. Subsequently in France, the constant cool environmental temperature of around 14-18°C found in subterranean caves and quarries was recognised as ideal for producing mushrooms at low cost and throughout all seasons. France soon became the major mushroom producing country by exploiting the availability of underground caves in the Paris region and along the Loire Valley (Atkins, 1966).

The Mushroom Industries in France, U.K. and U.S.A were well established by the end of the 19th Century (Duggar, 1925), although very little change in composting techniques had been made. The gradual acceptance of the mushroom into national cuisines encouraged mushroom growers and researchers to continue searching for methods to improve cropping. Considerable attention was directed, not only at compost formulation and composting technology, but also at providing the mushroom crop with a controlled environment.

A second stage of composting which proved advantageous for improving yields was discovered virtually by accident around the 1920's when mushroom growers in Pennsylvania, designed and adopted wooden growing houses accommodating a double row of fixed shelves 16-25 cm deep (F.L. Lambert, 1941). Compost that had not completed its thermogenesis in stacks and was then filled into such shelf beds continued to generate heat. This condition was termed 'sweating out' and it was soon realised that the higher, more uniform temperatures attained during this period of the fermentation, not

only produced a more selective substrate, but that invasions by insect pests and fungal pathogens were also considerably reduced. By 1930, this technique had become an established part of the composting procedure used by many growers, although its full significance was not appreciated until the work of E.B. Lambert, (1941). He identified particular areas of the traditional single phase mushroom stack where compost that was most selective for mushroom colonisation occurred. These were zones that remained between 50-60°C during the process. This technique of sweating out soon became an integral part of composting, not merely as a means to eradicate insect pests and fungal pathogens, but to produce a uniform selective compost. This process became known as the 'Pasteurisation or Peak-heat' phase of composting.

The popularity of the motor car in the 1920's and 30's initially presented a threat to the rapidly growing mushroom industry as the quantity of the basic raw material, horse-stable manure, began to diminish. This stimulated Waksman & Reneger (1934), Sinden (1938), Lambert (1941) and Stoller (1943) to look at alternative ingredients suitable for preparing mushroom substrates. These researchers approached the problem in a variety of ways employing a range of organic and inorganic ingredients. After considerable effort, they all reached similar conclusions. Mushrooms could be grown successfully on artificial or so called 'synthetic' compost mixtures using wheat straw as the basic component. This demonstrated that there was nothing unique in horse manure that could not be provided by other materials. This work also demonstrated that cereal straw was an essential constituent of mushroom composts, primarily as a source of carbon and not as many considered, a diluent necessary for its physical effect on improved aeration. Such findings began to quell the mysticism associated with mushroom production which up until this period was regarded as an art and not a science.

Pizer (1937) recognised that composts prepared by the traditional procedure varied considerably in appearance and physical condition. Composts that gave poor mushroom yields, irrespective of the degree of

straw degradation, were generally greasy by nature, were sticky to the hand and tended to bind together and recover little from compression. Such poor composts were not always the result of insufficient composting, overwatering or poor aeration. Turning his attention to the colloidal components present in composts, he found that the addition of calcium salts to compost materials flocculated or coagulated the colloids destroying their greasiness. Overall the addition of calcium, in the form of gypsum (Ca_2SO_4) created an environment more favourable for the development of resident microflora. Composting was more uniform as aeration was good and such composts were unlikely to contain residual 'pockets' of soluble carbon and nitrogen compounds which could later lead to the establishment of 'weed fungi'. As the physical nature of the composted substrate was more uniform, the mushroom mycelium became established more rapidly. After a number of years research, Pizer & Leaver (1947) stated that ground gypsum should be regarded as an essential ingredient to be included in all compost formulations. Mushroom growers, world wide, were quick to adopt this technique and an additional benefit of gypsum supplementation was found to be the buffering effect it had on the ammonia content of composts, reducing pH levels to below 8.0. The regular addition of gypsum to compost ingredients (generally 25-30 kilos per tonne of fresh materials) from the 1950's onwards, not only improved the physical condition of the substrate, but better yields of mushrooms were consistently obtained. With the adoption of this technique there was also a gradual tendency to reduce the Phase 1 composting stage on farms to below three weeks.

With the establishment of the Mushroom Research Association at Yaxley, Peterborough, in 1946, considerable effort was made by Edwards (1949) to introduce precision into compost formulation. He formulated compost mixtures which included dried blood as the main nitrogen source. Such formulations were employed successfully in many countries but little interest was shown by growers in the UK due to the increasing popularity of horse racing. This lead once again to the availability of large quantities of stable bedding as a low cost source of compost. Because of the tremendous

variability experienced in these manures and the fact that no grower systematically analysed his starting ingredients, the duration of Phase 1 remained upwards of two weeks. This was sufficient time for the resident microflora to transform the compost mixture, whether high or low in total nitrogen, into a substrate which was well balanced nutritionally for the mushroom.

Sinden & Hauser (1950), after many years of research in the U.S.A. were the first to outline a shortened technique of composting. Although this technique provided a somewhat less degraded substrate, mushroom yields were not only maintained but were steadily improved. Their analyses of experimental horse manure composts gathered over many years, revealed that the greatest loss of dry matter during Phase 1 composting occurred during the period between the 7th and 15th day. Yield, measured in weight of mushroom per unit area of bed space, reached a maximum in composts that had received only 11 days composting, when the decomposition of compost materials was still relatively small. Such a technique made a more efficient use of available materials and saved time, space and labour. The 'Sinden Short' method of composting, as it became known, was quickly adopted in America and Europe. Most growers conformed to a 7-14 day Phase 1 procedure on an open or covered yard followed by a 5-7 day Phase 2 in trays or shelves within a purpose built pasteurisation room. Later work by Sinden & Hauser (1953) emphasised that Phase 1 and Phase 2 composting were related parts of the composting process, dependant on each other, but at the same time differing in nature and function. Although it was appreciated that the biological activity of thermophilic micro-organisms was responsible for heating the stack to 65°C during Phase 1, temperature rises above this were attributed to chemical reactions. When temperatures exceeded 80°C in the stack two types of chemical reaction were said to occur:-

(a) oxidative reactions analagous to spontaneous combustion outlined by Browne (1933).

(b) chemical reactions affecting carbohydrates at high temperature resulting in the elimination of water from the sugar molecule (caramelisation).

Aware of the tremendous variability in mushroom compost starting materials i.e. stable bedding, Burrows (1951a,b) analysed a range of mushroom composts during Phase 1 composting. He showed that losses of nitrogen from mushroom composts appeared to depend upon at least three factors:-

(i) its starting total nitrogen level or carbon:nitrogen (C:N) ratio
(ii) temperature
(iii) the rate of initial conversion of nitrogen source to ammonia.

Compost with a high nitrogen content and consequently a low C:N ratio generally produced high levels of ammonia within the compost. Composts with a low total nitrogen content and therefore a high C:N ratio produced considerably less ammonia, and that which was evolved was rapidly assimiliated by compost microflora growing on the abundance of carbohydrates. Losses of nitrogen were also shown to be kept at a minimum by composting at 55°-60°C or using nitrogen sources which were less readily converted to ammonia.

Throughout the late 50's and early 60's, the 'Sinden Short' method of composting became the most popular method of composting used worldwide. However, Rasmussen (1963) after extensive experiments with pig and horse manure encouraged Danish mushroom growers to use a 16 day Phase 1 procedure as it was likely to produce more predictable results. Unlike 'Sinden Short' composts, which were usually 'active' before pasteurisation, composts prepared by the longer technique were well fermented, and generally free of ammonia taking only 3 days to complete the pasteurisation phase.

Hayes & Randle (1968) demonstated that the production of an ammonia free compost in Phase 1 can be aided by the supplementation of

compost mixtures with soluble carbohydrates such as sucrose or molasses. The improved yields obtained from these composts was explained by the dry matter conservation due to the shorter composting period and increased population of thermophilic bacteria. In the years that followed a number of proprietary activators appeared on the market specifically formulated i.e. containing readily available forms of both nitrogen and carbon, to accelerate the establishment of microbial populations during Phase 1 composting.

Single phase composting in controlled environments, to reduce composting time, conserve energy and materials while maintaining commercially acceptable mushroom yields, has been the goal of many researchers (Laborde & Delmas, 1969; Randle & Hayes, 1972; Smith, 1974: Smith & Spencer, 1976). While selective composts have resulted from single phase composting techniques, yield reductions of around 25% (Smith, 1983) are experienced and as a consequence of a reduced composting period bulk density is also reduced. As less compost can be compacted into a given growing container, and more importantly the growing house, a further reduction in mushroom yield can be expected. This reduction in yield can be addressed by adding N-rich supplements at spawning or casing (Laborde *et al*, 1979), but such additions can initiate microbial activity causing the compost to overheat, jeopardising the 'in-built' selectivity and the establishment of mushroom mycelium.

In 1970 compost experiments commenced in Italy to achieve fermentation, pasteurisation and mycelial colonisation (spawn-running) without practically moving the material. This was achieved sequentially in a purpose built composting 'tunnel'. This novel system of compost preparation (Derks, 1973), elements of which were to revolutionise the Industry in the following years, was termed the '3-Phase-1' system in a composting 'tunnel'. Supplements, i.e. animal manures and proprietary activators, were added to the compost starting ingredients and thoroughly mixed and wetted before being transferred to the tunnel. Using a special system of aeration, fresh air was pumped through the stacks from beneath. The fermentation period was claimed to be completed in 3-4 days without moving the compost and

pasteurisation was then allowed to follow immediately taking another 4 days. This idea of bulk composting was not original, as a similar method of under floor aeration had been suggested by Lambert & Davis (1934), but the idea of bulk colonisation of the substrate by mushroom mycelium was unique. While this three phase system was to suffer technological problems, Dutch researchers were quick to exploit the bulk pasteurisation and bulk spawn-running idea on a commercial scale (Gerrits, 1975). They retained the Phase I process of assembling materials on a covered yard and composting in windrow stacks for 8-10 days, but kept bulk pasteurisation and bulk spawn-running as independent stages. The outcome of this research led to the establishment of a large composting co-operative at Ottersum to supply Dutch growers with a reliable, fully colonised substrate (Vedder, 1977; Vedder, 1978). This system of compost preparation and bulk colonisation fitted in well with the shelf bed system of growing which had become the most popular method of growing throughout Holland in the late 60's and early 70's (Gaze, 1985). Although the majority of large farms throughout the world now use the bulk pasteurisation technique developed by the Dutch, the environmental conditions imposed on the composting substrate over two phases has remained virtually unaltered for thirty years.

A renewed interest into single phase controlled environment composting (Laborde et al, 1986: Gerrits 1987: Perrin & Gaze, 1987; Nair & Price, 1991; Noble & Gaze, 1994) has been prompted by proposed legislation to control the release of noxious odours to the atmosphere and toxic run-offs into water courses. Such experimentation is being pursued in bulk pasteurisation tunnels although it is inevitable that a preparation stage for mixing and blending of materials prior to filling will always be necessary.

1.3 The role of micro-organisms in compost

General microbiological surveys of organisms present in mushroom composts have been made by several workers including Hayes (1968), Laborde & Delmas (1969), Fordyce (1970), Imbernon & Leplae (1972), Lacey

(1973), Chanter & Spencer (1974), Rosenburg (1975, 1978), Fermor *et al.* (1979), Kleyn & Wetzler (1981), Amner *et al.* (1988) and Derikx (1991). All these reports give quantitative assessments of the populations of fungi and bacteria predominating within the substrate at different stages of its preparation.

During the initial stages of the composting process, the resident microflora, mainly mesophiles, preferentially utilises short-chain carbohydrates with the eventual production of microbial biomass, carbon dioxide, water and energy. Ammonia, also produced by the microbial activity, aids in the decomposition of the straw by softening its cell walls enhancing further microbial activity (Bels-Koning, 1962). The nitrogen requirement for the compost micro-organisms is smaller than the requirement for carbon, as generally one part of nitrogen is needed per 10-15 parts of carbon for cell growth and multiplication. This is obtained from both organic and inorganic nitrogen sources. Inorganic forms, such as nitrate or urea, generally have to be converted to ammonia before being reconstituted into microbial metabolism. Organic forms such as amino acids or protein, can be directly utilised by microflora, but they may also be subjected to deamination reactions resulting in the formation of ammonia.

As the compost temperature increases, a selection pressure is exerted on the microbial community which results in a progressive reduction in species type. Mesophilic bacteria are quickly replaced by thermophilic spore formers and mesophilic fungi are gradually succeeded by more thermotolerant types which grow and sporulate at 45°C and above. As composting progresses, there is a notable change in the actinomycete community, the high compost temperature favouring the development of more thermotolerant types, but it must be emphasised that there is no clear dividing line between the establishment of the groups. Some of the major groups of micro-organisms occurring in mushroom composts during its preparation are outlined in Table 1.

Generally on depletion of the readily utilisable carbon and nitrogen sources, there is a notable change in microbial groups, especially

Table 1. Major groups and succession of micro-organisms during compost preparation (After Fermor *et al*, 1985)

Mesophiles	Thermotolerant	Thermophiles
	Succession of micro-organisms →	
Bacteria *Flavobacterium* spp. *Pseudomonas* spp. *Serratia marcescens* Actinomycetes *Streptomyces* spp. *Nocardia* spp. *Faeni* spp.	*Pseudomonas* spp. *Bacillus licheniformis*	*B. coagulans* *B. stearothermophilus* *B. subtilis* *Thermoactinomycetes* spp. *Thermomonospora* spp. *Saccharomonospora viridis* *Streptomyces* spp.
Fungi *Mucor* spp. *Aspergillus* spp. *Penicillium* spp.	*Aspergillus fumigatus*	*Torula thermophila* *(syn. Scytalidium thermophilum)* *Chaetomium thermophile* *Humicola insolens* *Rhizomucor pusillus* *Talaromyces thermophilus* *Thermomyces lanuginosa*

thermophilic actinomycetes. These micro-organisms, possibly poor competitors for the short chain carbohydrates available in compost mixtures at the commencement of composting, produce cellulases capable of degrading cellulose and hemicellulose. This rapid increase in actinomycete populations produces a clearly visible and distinctive condition (sporulating colonies producing white flecks on the cereal straw surfaces) commonly referred to as 'firefang' by commercial growers. While this condition is a good indicator of the microbial succession taking place, it cannot be used on its own as an indicator that the compost is ready for inoculation with mushroom mycelium. Even during the later stages of pasteurisation, when in most cases thermophilic actinomycetes are well established, ammonia levels e.g. above 20 ppm will be the main factor limiting mushroom growth. In recent years, much attention has been paid to thermotolerant fungi which become predominant in mushroom composts in the latter stages of composting (the

so-called conditioning phase immediately following pasteurisation). It is thought that the presence of fungi such as *Torula thermophila* syn. *Scytalidium thermophilum* (Ross & Harris 1983 a,b; Straatsma *et al.*, 1989) play a key role in the rate at which mushroom mycelium colonises its substrate. Although upwards of 12% of the cellulose/hemicellulose fraction can be utilised by compost microflora during the preparation of a selective substrate (Smith, 1980), generally at the completion of composting, this fraction represents between 30-50% of the total dry matter remaining.

Micro-organisms colonising mushroom compost during the composting process can therefore be regarded as active agents in the preparation of a nutrient substrate. They fashion the chemical composition of the ligno-cellulosic mixture, change the physical nature of the ingredients, contribute directly to the ultimate nutrition of the mushroom and probably the most important factor of all, they produce a selective medium on which competitor organisms cannot readily grow.

1.4 Compost Selectivity or Specificity

Compost selectivity or specificity can be defined as the ability of a prepared substrate to promote a rapid colonisation of mushroom mycelium to the practical exclusion of other micro-organisms.

One of the earliest known references to mushroom cultivation and compost specificity can be attributed to Tounefort (1707) who worked at the Royal Academy of Science in France. Knowledge of composting was extremely limited at this time and the emphasis of work was directed at the design and preparation of mushroom beds, although the relationship between the production of a well structured uniform compost and eventual yield of mushrooms was clearly recognised. Very little work was directed at the chemical and microbiological aspects of organic substrate fermentation until Hebert (1892) began to investigate the chemical changes occurring in a typical farmyard manure heap. He observed that during the aerobic

decomposition of cereal straw and horse manure at high temperatures, up to 50 per cent of the total organic matter was lost. This large loss was attributed mainly to the breakdown of cellulosic material; and he concluded that the loss due to lignin degradation was small. An extensive synthesis of microbial protein from simple nitrogen compounds during the fermentation was also recognised, confirming the work of Deherain (1889). Both of these researchers suggested that a protein-lignin complex accumulated during composting, a complex which was extremely resistant to further attack by micro-organisms.

Much confusion originated between 1900 and 1930 over the content of organic matter in composts. Waksman & Stevens (1930) began to clarify the chemical changes occurring in composts following a critical study of the analytical methods used to determine the nature and abundance of organic materials in soils. Many previous workers had recognised a definite dark coloured fraction of soil organic matter which they termed 'humus', a fraction that had been commonly associated with soil fertility. This fraction had been regarded as an intermediary product resulting from the decomposition of plant remains and manures, the final products of decomposition being ammonia and carbon dioxide. Waksman did not regard these dark coloured substances as intermediary decomposition products as they were stable and resisted further attack by micro-organisms. These products were the result of both direct and indirect decomposition of plant residues together with the products of microbial synthesis. Analysis of 'humified' organic matter soon revealed that nitrogen was firmly complexed to this fraction such that strong acid or alkali treatment could not remove this nitrogen completely, without destroying the whole complex.

Waksman & Magrath (1931) turned their attention to the microbiological and chemical processes involved in the decomposition of farm yard manure prior to and after its inoculation with mushroom mycelium. They showed that, before inoculation, hemicellulose and cellulose are decomposed with equal rapidity, whilst the lignin content was hardly affected. They also identified an association between lignin and protein with the formation of a

'lignin humus complex' which was resistant to further attack by micro-organisms. Microbial activity therefore resulted in the accumulation of organic nitrogen complexes and a decrease in free ammonia and soluble organic matter. The removal of the readily decomposable complexes prevented other organisms from rapidly colonising the remaining composted materials. The most interesting results were observed in the changes that took place in the relative concentration of lignin and protein or 'lignin humus complex' as it was to become commonly termed after composting had been completed. During composting, a gradual and continuous increase in this complex was noticed. After colonisation of the substrate by mushroom mycelium, there was a rapid reduction in both the protein and lignin content of this complex which suggested that the mushroom itself was utilising these complexes both as a source of energy and for further synthesis of mushroom biomass. These findings were to form the basis of further research studies in the years that followed.

Stoller (1941) also postulated that the lignin component of mushroom composts precipitated or closely bound the nitrogeneous material during composting. He claimed that while this closely bound nitrogen was resistant to further attack by compost micro-organisms, mushroom mycelium had the ability to utilise it. He also noted that other substances besides lignin were capable of complexing nitrogenous compounds within their structure and found that supplementation of mushroom composts with tannins improved sporophore yield. The precipitation or binding process of nitrogenous compounds he termed 'coprination' and the materials responsible for this chemical reaction he termed 'coprinating agents'. He also claimed that such complex chemical agents could be characterised as having polyhydroxy groups within their structure.

Gerrits and co-workers (1967) carried out exhaustive studies of organic matter degradation during the composting of stable manure and also determined the subsequent utilisation of organic matter by mushroom mycelium (Gerrits, 1969). Estimation of lignin, using an acid digestion procedure (72% sulphuric acid) first outlined by Ritter, Seborg & Mitchell,

(1932), revealed that this fraction contained significant quantities of nitrogen although pure lignin is a non-nitrogen containing aromatic polymer (Crawford, 1981). This fraction they called the 'nitrogen-rich lignin humus complex'. This apparent 'nitrogen contamination' of lignin during composting could possibly be explained by humic substances rich in nitrogen becoming complexed with residual lignin during the acid extraction procedure. However one explains the insoluble nitrogen linked to the lignin fraction, it is quite clear that lignin is degraded during the colonisation by mushroom mycelium. As the nitrogen content of the 'nitrogen-rich lignin humus complex' has been shown to remain constant, from the introduction of the mycelium until the end of cropping, it is assumed that the whole complex, including the incorporated nitrogen is being utilised (Gerrits, 1969), confirming the findings of Waksman & McGrath (1931). It was estimated that between 40 and 70 per cent of nitrogen utilised by the mushroom came from the nitrogen firmly bound to the lignin complex. Some years later, Grabbe (1972), demonstrated that in some horse manure composts, as much as 80 per cent of the insoluble nitrogen present in composts could be isolated from this complex. As such forms of nitrogen appeared to be resistant to bacterial decomposition, but available to *Agaricus*, the substrate had a certain amount of specificity conferred upon it. Micro-organisms colonising such substrates during the course of the fermentation could therefore be regarded as active agents in the preparation of a nutrient medium as they fashioned the chemical composition of the substrate, changed its physical nature and made the growth of competitive micro-organisms more difficult.

Hayes and Randle (1969) discovered that supplementation of composts during Phase 1 with soluble carbohydrates, accelerated the composting process and decreased the presence of ammonia. The improved yields obtained from these composts was shown to be associated with an increased population of thermophilic bacteria and a conservation of dry matter. These workers suggested that by encouraging the growth of thermophilic bacteria, the population of thermotolerant actinomycetes was reduced and since such organisms are known to utilise cellulose,

hemicellulose and lignin, a net conservation of nutrients available for the production of mushroom fruitbodies was achieved. Chanter & Spencer (1974) compared the development of microbial populations in a traditional compost stack and in compost placed in a revolving drum under controlled environmental conditions. While both systems encouraged a mixed population of micro-organisms, the controlled and relatively uniform high temperature within the drum (55-60°C) promoted the development of large numbers of thermophilic bacteria and fewer thermotolerant fungi. An ammonia free selective substrate could therefore be produced more quickly.

Stanek & Zatecha (1967) studied the microbial groups involved in mushroom composting and indicated that many thermophilic actinomycetes produce growth promoting substances such as pantothenic acid; nicotinic acid and thiamine which had been shown by Treschow (1944) to stimulate growth of mushroom mycelium. Stanek (1972) later found that growth of mushroom mycelium was improved and the compost remained free of fungal contaminants when it contained both thermophilic bacteria and actinomycetes. He also demonstrated that thermophilic bacteria have difficulty in decomposing cellulose and hemicellulose, but it appeared that filtrates from the cultures of bacteria stimulated actinomycetes which could degrade these polymers. Similarly, filtrates from actinomycetes stimulated growth of bacteria, producing polysaccharides which were claimed to be more efficiently utilised by the mushroom than simple monosaccharides.

Smith (1974, 1977) investigating rapid composting techniques to induce selectivity into defined compost mixtures found that an efficient utilisation of soluble compounds by thermophilic micro-organisms was essential. Later, Smith & Spencer (1976, 1977), investigating the limitations of nitrogen supplementation in short duration mushroom composts found that compost formulations with low total nitrogen levels at the start of composting i.e. between 1.0 - 1.4 per cent of the dry matter, gave the best results. More significantly, analyses of the nitrogen complexed to the lignin fraction revealed that the higher yielding mixtures, although having low starting nitrogen levels, retained up to 50 per cent of their nitrogen in this insoluble form. In

unselective mixtures, where undesirable fungal contaminants predominated, the level of this complexed nitrogen was only 37 per cent after composting. These trials indicated that there is a limit to which microbial processes can be hastened to convert soluble forms of nitrogen into insoluble forms and consequently to prepare selective composts by a short duration technique precision of formulation is essential. In later trials, variable amounts of sucrose were added to defined compost mixtures at the start of composting to increase bacterial populations and to investigate whether an increased activity would lead to an increase in lignin-bound nitrogen. Analyses of sucrose-supplemented composts revealed no apparent increase in total nitrogen or lignin-bound nitrogen at spawning with increasing sucrose levels, but those substrates receiving the higher levels of supplementation gave the highest mushroom yields. The overall conclusions confirmed the findings of earlier researchers, that selective composts should have a high proportion of their nitrogen as lignin bound nitrogen and that thermophilic micro-organisms and the biomass that they create may also contribute a source of insoluble nitrogen as microbial protein.

Eddy & Jacobs (1976) isolated the 'dark surface layer' which accumulates on the internal and external surfaces of straw during the composting process. This dark material, which builds up during the composting process on the internal and external surfaces of the cereal straw component, has an amorphous matrix with the staining characteristics of bacterial polysaccharide. This material was shown to disappear rapidly during compost colonisation by *Agaricus* effecting a change in compost colour from dark to light brown. These workers also suggested that the mushroom derives a large proportion of its nutriment from the microbial biomass built up by the rapid growth and turnover of thermophilic microflora.

Fermor & Wood (1981), Sparling, Fermor & Wood (1982), Fermor (1983) and Fermor & Grant (1985) clearly demonstrated that the mushroom mycelium produces a number of extracellular enzymes capable of attacking the cell walls and contents of dead bacteria and fungi.

Ross (1978) and Ross & Harris (1982) also investigated factors

affecting compost selectivity. They noted that the temperature during phase II composting significantly affected the rate at which ammonia produced by microbial activity is lost to the atmosphere. At temperatures between 40°-45°C ammonia disappearance was the fastest, although the majority of composts produced in the 45°-53°C range proved to be high yielding. At temperatures in excess of this range ammonia was more difficult to remove and generally persisted no matter what length of composting time was allowed. During the course of their studies Ross & Harris (1983b) also isolated compost microflora from completed Phase 2 composts and used them to inoculate experimental composts at the commencement of Phase 2. The addition of such inocula speeded-up the disappearance of ammonia. A number of isolates were tested separately and certain thermotolerant fungi were recognised to be particularly effective. Further investigations were directed at destroying the in-built selectivity using heat and antimicrobial agents such as chloroform and ethanol (Ross & Harris, 1983a) and then restoring it by repeating a Phase 2 stage around 45°C. This operation was greatly speeded up when composts were inoculated with the thermophilic fungus *Torula thermophila*. From these observations, they concluded that to remain selective, a mushroom compost must have a biomass which is relatively intact and consist of live or dormant microflora at the time of inoculation with *Agaricus* mycelium.

Smith (1983), continued to evaluate the short duration composting concept for commercial exploitation and developed a single phase composting procedure which harnessed the heat from the developing microbial populations to achieve both pasteurisation and conditioning temperatures in a single phase operation. After a brief pasteurisation period between 60-65°C, the compost temperature was reduced to 53-55°C for the establishment of thermophilic bacteria and actinomycetes. The resulting substrates from this procedure, very light in colour, were notably free of thermophilic fungi. Ammonia free selective substrates were produced in 5 days, although yields were about 25% less than from commercial composts. Laborde, who had also pursued the short duration composting concept for

many years (Laborde & Delmas, 1969; Laborde *et al.*, 1979) suggested that the low yields experienced from such composts were associated with a reduced microbial status and that yields could be increased to an acceptable level by supplementation of the compost at spawning or after colonisation, with nitrogen rich additives (Laborde, 1980).

Straatsma *et al* (1989), after evaluating work on the presence of thermophilic fungi in mushroom composts, isolated *Scytalidium thermophilum (Torula thermophila)* and studied its stimulatory effect on growth rate and yield of *Agaricus bisporus*. This organism belongs to the same taxonomic group as *Humicola* spp. and was shown by Bels-Koning (1962) to have a beneficial effect on compost selectivity and productivity. Mycelial growth of *Agaricus bisporus* in sterilised compost was claimed to be strongly stimulated by pre-incubating the compost with *S. thermophilum*. This stimulatory effect was not claimed to be species specific. The conclusions by both Bels-Koning (1962) and Straatsma *et al* (1989) were that the precolonisation of mushroom compost by thermophilic fungi such as *S. thermophilum* was essential for successful colonisation by mushroom mycelium, and that composting temperatures, as shown by Ross (1976), should be maintained around 45°C during Phase II to allow the natural build up of such organisms.

1.5 The humus fraction of mushroom composts

While insoluble forms of nitrogen, i.e. as nitrogen linked to the lignin complex or as microbial protein, have been identified as important sources of nitrogen for mushroom nutrition, there is still much confusion over whether these forms of nitrogen are distinct or one and the same. The harsh acid digestion procedure commonly employed to isolate the total lignin fraction from mushroom composts always produces a dark material with nitrogen as an impurity, the so called 'lignin-nitrogen'. As lignin is known to be nitrogen free (Crawford, 1981), this impurity may be regarded as an artifact of the extraction procedure and it is highly likely that this nitrogen is of microbial origin. While much attention has been given by mushroom researchers in the

past to insoluble forms of nitrogen closely bound to the lignin fraction, a more reliable approach to identify insoluble forms of nitrogen accumulating during the composting process is to employ the well established method of humus extraction of soils with dilute alkali. The humus fraction comprising the dark surface layer of cereal straw composts can be isolated and then further subdivided into humic and fulvic acid fractions which can be separately analysed for their nitrogen content.

Humic acid, the dark coloured material extracted from soil, by dilute alkali and precipitated by dilute acid, appears to be a complex polymer of phenolic units with linked amino acids, peptides and other organic constituents. This fraction constitutes 50-80 per cent of soil humus (Martin 1971). Grabbe (1972), showed that during composting and pasteurisation of horse manure composts, a high proportion of the nitrogen is fixed into the humus and lignin fractions and at the completion of composting these two fractions make up 60-70 per cent of total organic matter, a total level not dissimilar to that found by Martin & Haider (1971). Grabbe (1972) also analysed the phenolic fractions within the humus fraction and clearly distinguished between phenols originating from lignin and those of microbial origin.

Wain (1981), continuing the work of Eddy (1976), extracted the dark surface material that accumulated on straw surfaces during composting and analysed its constituents. Whereas, Eddy used a lengthy Soxhlet extraction procedure based on the use of dilute sodium hydroxide to extract this fraction, Wain used a short sonication technique which was claimed to be more reliable as the chemical composition of the extracted material was not altered. Analyses of the extracted material showed it to contain approximately 40% carbohydrate, 25% protein and 7% phenol and such material comprised 30% of dry compost at spawning. Two fractions were detected, an alkali soluble/acid insoluble fraction and a debris component insoluble in alkali. Determining the utilisation of these two compost fractions by the mushroom mycelium during colonisation and subsequent cropping is difficult to assess when the mycelial biomass itself, as a proportion of the total compost dry

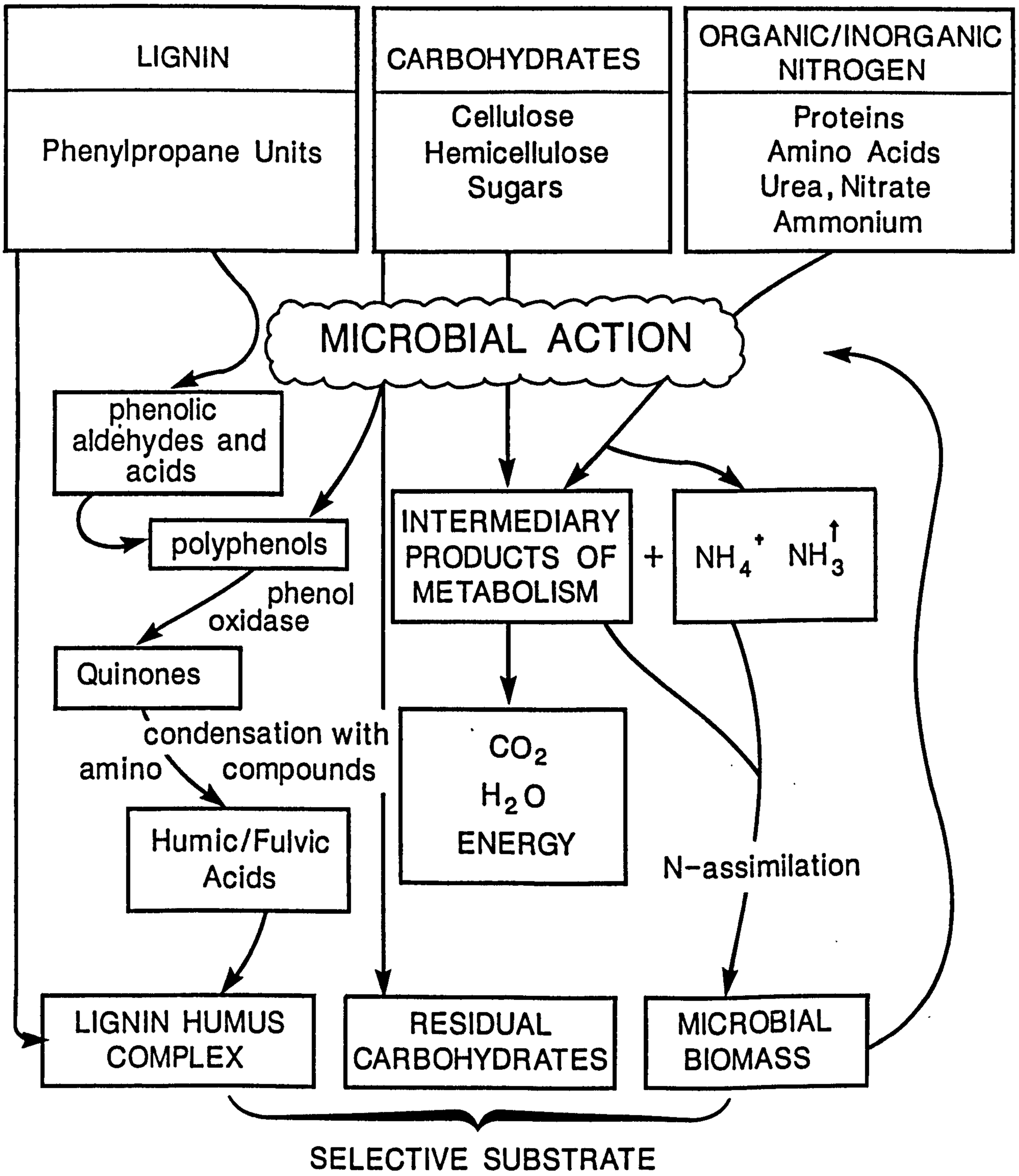
matter, can increase to above 10% during this period. Nevertheless Wain (1981), using a calculated mushroom interference factor based on fungal biomass determinations made by measuring laccase activity within a substrate (Wood & Goodeneough, 1977), attempted to correct analytical data gathered during the colonisation and subsequent cropping period. Large proportions of both these fractions were claimed to be utilised by the mushroom. Examination of the individual fractions using electron microscopy suggested that the debris fraction contains the majority of the microbial cells and the alkali soluble/acid insoluble fraction, an amorphous material. Further analysis of the carbohydrate and phenol monomers suggested that these components were of microbial origin rather than directly derived from straw components. Wain concluded, like many researchers before him, that the humus-biomass complex resembled humic acids that can be isolated from soil viz. both are composed of phenolic substances and closely associated with protein, dark brown to black in colour, soluble in alkali and precipitated by strong acid. Humic acid production in composted wheat straw substrates and its ability is to stabilise protein against microbial degradation (Haider, 1976) would appear to be a very important contributing factor towards compost specificity for the culture of the edible fungus *A. bisporus.*

A schematic representation of the chemical transformations occurring in mushroom composts as a result of microbial action, incorporating knowledge of the synthesis of humic substances (Stevenson, 1982) is shown in Figure 1 (Smith, 1993).

1.6 Extracellular enzyme activities of Agaricus bisporus in composted substrates

The most favourable temperature for colonisation of composted substrates by *Agaricus bisporus* and many other *Agaricus* species is 22°-25°C. At these temperatures, mycelial colonisation of the substrate is normally completed within 10-14 days. Chemical analyses of the substrate and measurement of the degradation rate of radio-labelled compounds within the substrate has shown that the mycelium can degrade cellulose, hemicellulose, protein and lignin (Wood & Leatham, 1983; Durrant et al, 1991). Fermor & Wood (1981) and Fermor & Grant (1985) have shown that *A. bisporus* can also degrade microbial biomass efficiently. Studies at H.R.I. initially concentrated on the microbial cell and debris fraction of the cereal straw coating and its relevance to mushroom nutrition. The most commonly occurring organisms found in well prepared composts were isolated and characterised (Fermor *et al*, 1979). *A. bisporus* was shown to be capable of growth on a range of heat-killed bacteria, both Gram-positive and Gram-negative, embedded in a mineral salt base agar, the bacteria being the only source of carbon and nitrogen (Fermor & Wood, 1981). Cleared zones in the agar beyond the colony perimeter indicated the production of bacteriolytic extracellular enzymes. Crude compost extracts taken from compost samples at the peak microbial degradation time i.e. between 14 and 28 days after inoculation with *A. bisporus*, showed high levels of β-*N*-acetyl glucosaminidase activity (Fermor & Wood, 1981), with peptidoglycan fragments forming the main degradation product. Further assays on partially purified bacteriolytic enzymes also showed muramidases or `lysozymes' are produced specifically for bacterial cell wall degradation (Grant, *et al.*, 1984). *A. bisporus* has also been shown to degrade the mycelium of the dominant thermophilic fungus found in compost i.e. *Humicola insolens* var. *thermoidea* (Fermor & Grant, 1985) as well as a range of *Actinomycete* spp. (Atkey and Wood, 1983). The microbial biomass in horse manure/wheatstraw compost immediately prior to spawning has been estimated, from direct

Figure 1. Chemical changes occurring in mushroom substrates during the composting cycle (After Smith, 1993)



counts and biochemical tests to be no more than 2% of the compost dry matter (Sparling *et al.*, 1982). If *A. bisporus* utilised the total amount of microbial biomass available, then the maximum contribution of the microbial biomass to the mushroom biomass would be less than 10%. This suggested that mushroom biomass derives the bulk of its C-nutrition from straw carbon and not from microbial biomass. In addition, microbial biomass may be a concentrated source of nitrogen and other minerals, and provide through the presence of polysaccharide polymers, a means of holding large quantities of water within the substrate (Eddy and Jacobs, 1976; Wood & Fermor, 1985).

The most abundant extracellular enzyme produced by *A. bisporus* is the phenol oxidase laccase. This enzyme has received considerable attention (Turner *et al*, 1975; Wood & Goodenough, 1977; Wood 1980). Laccase is produced from the onset of mycelial colonisation and its activity declines at the onset of fruitbody production when extracellular endocellulase activity increases significantly. Wood (1979), has shown a direct relationship between laccase activity within the substrate and total fungal biomass present prior to fruiting allowing a simple assay method to determine fungal biomass in both solid and liquid substrates. Claydon *et al* (1988), demonstrated that fruitbody biomass or crop yield is correlated with surges in extracellular endocellulase production within the substrate. Smith *et al* (1989), confirmed these findings and also observed that laccase production in very deep layers of compost during mushroom production reached levels four times that found in conventional shallow trays of compost. The role of laccase in the growth and development of mushrooms on composted substrates is still uncertain, although Latter & Burges (1960), demonstrated that laccase is capable of degrading humic substances. This enzyme has also been associated with lignin degradation and maybe also involved in the detoxification or degradation of phenolic complexes accumulated during the composting process (Wood & Smith, 1986), complexes which could be part of the humic acid fraction.

The nutritional strategy of *A. bisporus*, revealed by the identification of the extracellular enzymes it produces within the compost, is highlighted in Table 2.

Table 2. Extracellular enzyme activity demonstrated to be produced by *Agaricus bisporus* (After Wood & Smith, 1986).

Enzyme	Likely substrate in compost	Possible products and/or assimilates
Laccase	Lignin or phenols	?
Endocellulase Exocellulase β-glucosidase	Cellulose and Cellobiose	Cellobiose and Glucose
Cellobiose oxidase	Cellobiose	Glucose
Xylanase	Xylan	Xylose
Proteases	Protein	Amino Acids and/or peptides
β-N-acetyl muramidase (lysozyme)	Peptidoglycan (bacterial cell walls)	Peptidoglycan fragments
β-N-acetyl glucosaminidase	Microbial wall fragments	Peptidoglycan fragments
DNAase	Microbial/plant DNA	Sugars/nucleotides
RNAase	Microbial/plant RNA	Sugars/nucleotides
Lipase	Microbial/plant lipids	Fatty Acids
Laminarinase	β-1,3 glucans	Glucose

1.7 Mushroom mycelium growth rate as an indicator of compost selectivity

Although a great deal is now known regarding the microbial groups dominating mushroom composts and how they utilise the readily available carbon and nitrogen sources of a compost mixture, compost selectivity or specificity is still a loosely used and not fully understood term. To most growers and researchers a selective compost is a substrate that is rapidly colonised by mushroom mycelium with the absence of growth of competitor 'weed' moulds. It has been assumed that a substrate with these properties is productive and yet there is little published work to substantiate this.

The work of Straatsma *et al* (1989) concluded that the presence of fungi such as *Torula thermophila* (syn. *Scytalidium thermophilum*) in mushroom composts was important for the successful colonisation of the compost by the mycelium of *Agaricus* and the ultimate yield of mushrooms. These conclusions were based on the comparison of growth rate measurements ('race-tubes') of mushroom mycelium in composts that were (a) as prepared (b) autoclaved to kill resident micro-flora and (c) autoclaved substrates pre-inoculated with *S. thermophilum* and other organisms. It was found that pre-incubating autoclaved compost with 1% of untreated compost or inoculating *S. thermophilum* restored the rate of mycelial growth to the level recorded in the untreated control. Pre-incubation with the bacterium *Bacillus licheniformis* was shown to have an adverse effect on growth rate. Selectivity was therefore judged on mycelial growth rate alone. As the growth rate in autoclaved substrates was only (3-4 mm d^{-1}), it was assumed that the growth of mushroom mycelium was poor in comparison to the unautoclaved series which recorded growth between (8-9 mm d^{-1}). This assumption was based only on the macroscopic evidence that there was no difference in mycelial density other than subtle morphological differences in mycelial stranding (shorter and curlier); an alternative interpretation of the slower growth in autoclaved substrate could be explained by the fact that the mushroom did not have to compete for nutrients with resident microflora

which were killed by the autoclaving procedure. Measurement of the extracellular enzyme laccase produced by the mushroom during vegetative culture and shown by Wood (1979) to be produced in direct proportion to fungal biomass would have been an ideal assay procedure to clarify whether the presence of thermotolerant fungi at the completion of composting actually resulted in a denser mushroom mycelium. Very recent work by Straatsma et al (1993), using both liquid and solid agar media, to measure growth parameters of *A. bisporus* mycelium influenced by *S. thermophilum*, shows that this organism merely affects the radial extension rate of growth rather than having a positive effect on the specific growth rate. In contrast to this work, Weigant *et al* (1992), also using *S. thermophilum* in compost, claim that the growth promoting effect that this organism has on mushroom mycelium can be explained by increased carbon dioxide production which favours increased growth of *A. bisporus* mycelium. This has been disputed in the recent work of Straatsma *et al* (1993).

1.8 Concluding comments on compost selectivity

Three main conclusions may be drawn from this brief review of the preparation of composted substrates for *A. bisporus* and the factors associated with compost selectivity.

(1) *A. bisporus*, in non-axenic culture, is a very poor competitor for soluble nutrients. For this reason, it is essential to reduce such nutrients, by microbial action, to a level which does not encourage the establishment of other mesophilic fungi.

(2) During the composting process, insoluble forms of nitrogen are produced, either directly or indirectly by microbial action. There is much evidence to suggest that nitrogen complexed to the humus fraction and also as microbial protein is important in mushroom nutrition.

(3) At the completion of composting (i.e. at the time of spawning) the microbial biomass has to remain relatively intact to confer specificity and to allow efficient colonisation by *A. bisporus*. There is still some confusion over which groups of micro-organisms should predominate at this stage, and their contribution to substrate specificity.

This thesis attempts to identify chemical indices within the compost, built up during the course of the two traditional phases of composting, which relate to selectivity favouring mushroom colonisation and ultimate yield.

CHAPTER 2 MATERIALS AND METHODS

2.1 Composting materials

Unless otherwise stated the compost used in all experiments was a wheat straw compost (Randle & Flegg, 1985) which was prepared from deep litter chicken manure (normally between 3-5% N dry wt.), a commercial activator Sporavite, rich in molasses (25% soluble carbohydrate, 6.25%N) and Gypsum (calcium sulphate). This H.R.I. formulation was subjected to the traditional procedure of two distinct composting phases. Phase I composting was carried out in windrow stacks and took 10/14 days and the pasteurisation or Phase II stage was normally completed in trays within 6-7 days. The prepared compost generally had a nitrogen content close to 2%, a pH between 7.0-7.5 and a moisture content between 72-74%.

2.2 *Agaricus* species

A number of *Agaricus bisporus* strains were selected from the HRI culture collection together with a number of closely related wild species. These were chosen for their ability to colonise traditionally prepared mushroom compost. The total list of *Agaricus* species employed in these trials for use as indicators of compost quality or selectivity are listed as follows :-

A. bisporus	Somycel 649*	Horst U1*
	Horst U3*	Sinden-Hauser A9.3*
A. bitorquis	Horst K32*	Horst K26*
	W19 (tropical origin)	DDR 36-2 (German origin)
	W20 (tropical origin)	
A. arvensis	DDR 30-4 (German origin)	
A. brozei	W28	
A. campestris	W1A	
A. macrosporus	W7	
A. nivescens	WH23	

* Commercial strains. All other strains are designated with 'HRI culture collection' code

2.3 Design of bench-scale composting units

In order to produce a range of composts with increasing degrees of degradation, two composting units were built to accommodate three 2l Multi Adaptor Flasks (MAF). Each unit consisted of a fibre glass water bath enclosed in an insulated wooden box (Figure 2). Three holes were cut in the fibre glass lid at a suitable diameter such that each MAF flask could be easily seated and immersed up to its neck in water. Where composts were of low bulk density, the flasks were forcibly held beneath the water line, up to their necks, using restraining straps.

The 2l MAF flasks chosen were capable of holding 1 kg of compost and stainless steel platforms were made to separate the compost from any liquid runoff that may result from the extended composting period. To maintain an aerobic environment in the compost, each flask was independently aerated with humidified air by passing air via a 250 ml water-bubbler using a small 'Whisper 200' pump. An air flow sufficient to give 3 complete air changes per flask per hour (i.e. 6 litres) was achieved using rotameters (1100 Series - 25l/hr capacity; Fischer Controls Ltd, Croydon). Each rotameter was set to deliver 15l/hr and was periodically switched on using a time clock (2 mins in every 5 mins). A thermostatically controlled heater stirrer unit was placed at one end of the water bath and also switched on in synchrony with the aeration.

Unless otherwise stated, 6 MAF flasks (i.e. 2 MAF Composting Units) were used in each experiment and 800g of fresh weight substrate was compacted above the stainless steel platform in each flask. On completion of an extended composting period (see 2.4), the compost in each flask was checked for its ammonia content (see 2.6.3) and provided its concentration was below 10 ppm, the compost was allowed to cool to 25°C. The contents of each flask were accurately weighed before emptying to determine the fresh weight of compost remaining. The contents of the two flasks were then combined, well mixed, and the pH of the compost measured. Normally, a fresh weight compost sample between 50-100 g was taken and freeze dried for later analysis and for the determination of moisture content.

2.4 Substrate preparation and modification

To ensure consistency between experiments, a standard H.R.I. compost formulation was used (see 2:1), based on fresh wheat straw to which chicken manure and a commercial activator (of known carbon and nitrogen content) had been added (Randle & Flegg, 1985). To produce a wide range of substrates with varying degrees of degradation from this formulation, further compost samples were taken, after the traditional two phases had been completed, and subjected to further periods of composting. Low Phase 2 composting temperatures (below 50°C) are regarded as critical for compost specificity to encourage thermotolerant fungi i.e. *Scytalidium thermophilum*, therefore a temperature of 45°C was chosen as one treatment for prolonged composting. To produce composts in which thermotolerant fungi were less dominant and thermophilic actinomycetes well established, a prolonged temperature treatment at 55°C was also chosen.

Using 2 MAF units (i.e. 6 flasks), with water bath temperatures set at 45°C and 55°C, composts were prepared over a 1, 2 and 3 week period, such that in each experiment 7 compost types were available for testing with *Agaricus* strains (i.e. a control compost and composts prepared over 1, 2 & 3 additional weeks at 45°C and 55°C). In all cases where composting was prolonged, soluble carbohydrate (glucose) was added to the substrate at 0.9 per cent of the fresh weight to give a soluble carbohydrate level close to 3% of the dry matter at the start (this assumes that all composts have a moisture content of at least 70% at the time of supplementation). This additional carbohydrate provided the naturally occurring microflora with readily available carbon, widening the compost C:N ratio. It is essential that at the completion of composting, traditionally prepared substrates have a very low soluble carbohydrate level, otherwise contamination by weed fungi (invading moulds) rapidly occurs, but an unnatural forced extension of the composting period beyond this level without readily available carbon could encourage the

resident micro-organisms to breakdown protein as a carbon source. This could result in the production of ammonia once again, and consequently the loss of compost selectivity. Therefore to produce ammonia free substrates for testing on a weekly basis this precautionary method of soluble carbohydrate addition to composts at the commencement of a prolonged composting treatment was followed in the early experiments.

2.5 Mushroom inoculum (spawn) preparation

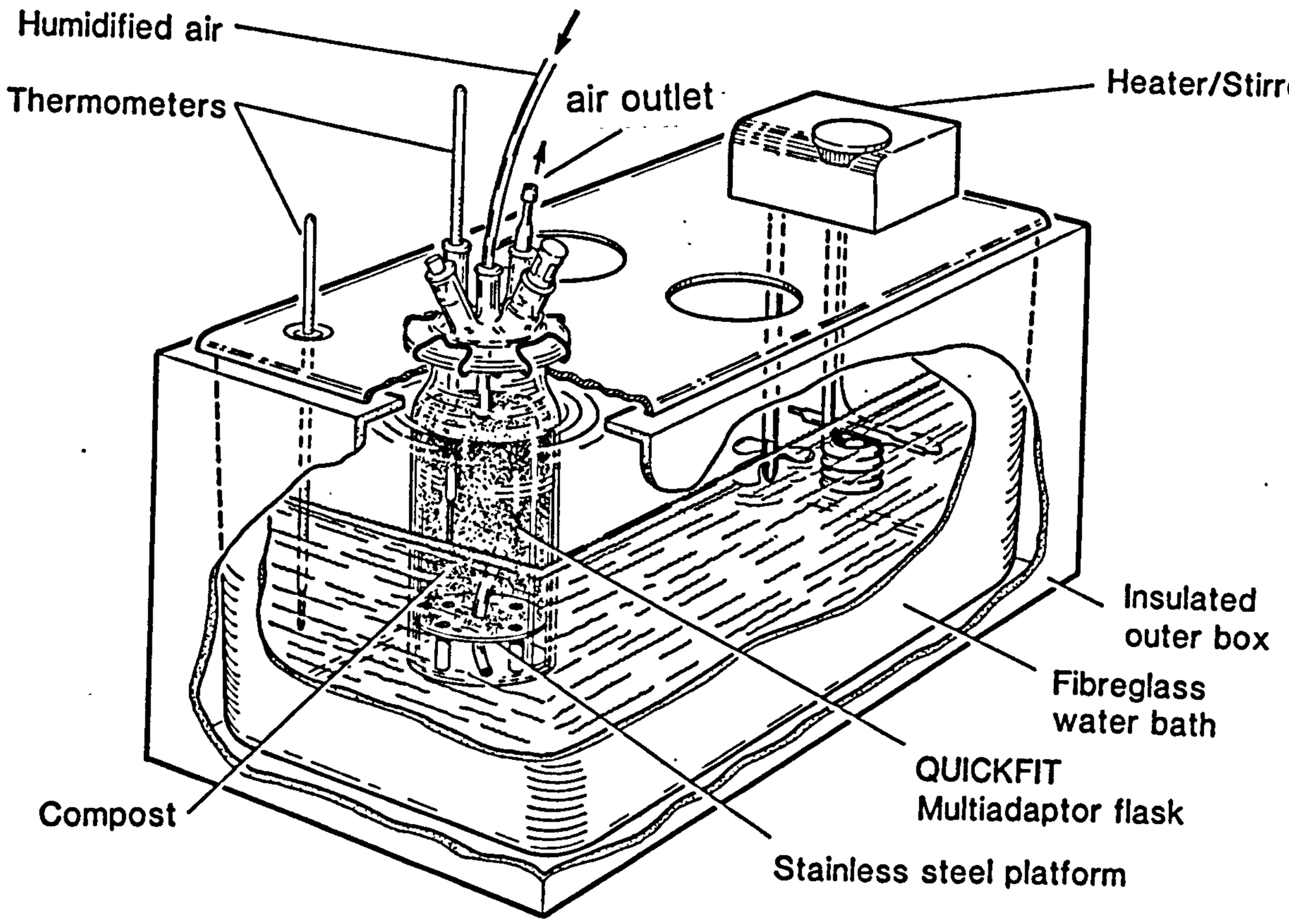
In all the trials described in this thesis, rye grain was used as the substrate for the mushroom mycelium used as an inoculum. This grain inoculum was prepared in the commercial manner by boiling in water to increase the moisture content of the grain from around 10 per cent to 40 per cent by weight. Gypsum (calcium sulphate) 2 per cent by weight and powdered chalk (calcium carbonate) 0.5 per cent by weight were then added to the swollen grains to adjust the pH and reduce grain adhesion. The supplemented grain was filled into 300 ml glass jars (aluminium screw lids) and autoclaved for 2h at 121°C and 15 psi. On cooling, each jar was inoculated with 2/3 agar plugs taken from an actively growing culture and incubated at 25°C. Frequent manual shaking of the cultures, every 2/3 days after 10 days of static culture, produced a 'flowable' product (i.e. no grain adhesion) in 3/4 weeks. The commercial strains used in these trials were also available on rye grain.

2.6 Methods of compost analysis

2.6.1 Moisture, pH and total ash determination

The moisture contents of all compost samples were determined by drying duplicate samples (c. 20g) of compost for 5/6 days to constant weight in a Freeze Dryer. After dry weight determination, both samples were combined, ball milled to a fine powder and then stored in air-tight glass

Figure 2. Multi-adaptor flask fermentation unit.



bottles for further analysis. This method was used because determining moisture content by drying fresh compost samples to a constant weight in an oven can result in the loss of nitrogen forms such as ammonia, leading to inaccurate determinations of total nitrogen.

To determine pH, as a standard practice, duplicate samples (c. 25g) of fresh weight compost were taken at the completion of composting and 100 ml distilled water added to form a suspension in a beaker. After a thorough mixing, the suspension was left to stand for 15 minutes before pH determinations were made using a Philips PW 9418 pH meter.

To determine ash content, duplicate samples of freeze-dried compost (0.5g) were weighed out into porcelain crucibles and ignited in a Griffin-Electric Furnace at 600°C for a period of 12/14 hrs. The total ash content was determined by averaging the residual sample weights.

2.6.2 Total nitrogen

Total nitrogen was determined by a micro-Kjeldahl method first outlined by O'Neill and Webb (1970). Duplicate samples of freeze-dried compost (0.1g) were placed in 20 ml Excelo tubes with 2 ml of digestion mixture (i.e. N-free sulphuric acid containing 0.5 per cent Selenium powder). Tubes were heated in an aluminium block until digestion was complete (normally 2hrs at 250°C; 4hrs at 380°C; 2hrs cooling). On cooling, the tubes were made up to 20 mls, shaken and then filtered. Total N was then measured using a Technicon Auto Analyser (Technicon Corporation, Tarrytown, New York).

2.6.3 Ammonia

Concentrations of ammonia in prepared composts at spawning were determined using a Draeger Gas Detector Pump with appropriate sample tubes. Each tube (CH 20501), was inserted in the Draeger bellows pump and by operating the prescribed number of suction strokes (10) an air sample was sucked through the tube. In the presence of ammonia the indicator layer

changes from a yellowish-orange colour to blue and the gas concentration in parts per million can be determined by reading the length of discolouration within the sample tube.

2.6.4 Ammonium nitrogen

Duplicate samples of freeze-dried compost (0.3g) were placed in 250 ml conical flasks and shaken with 25 ml of 2M KCl on a water bath set at 25°C for 2 hrs to extract the ammonium ion. Samples were then filtered through Whatman No.4 filter paper. The supernatants were made up to 50 ml and 20 ml was taken for N analysis on the Technicon Auto Analyser.

2.6.5 Soluble carbohydrate

Soluble sugars in compost samples were determined colorimetrically by a method first developed by Dubois *et al* (1956). Simple sugars react with a phenol sulphuric acid mixture to produce a distinct orange/yellow colouration which is stable for several hours. For compost analysis, duplicate freeze-dried samples (0.25g) were treated with 3 x 25 ml volumes of 80 per cent ethanol and the extracts filtered and bulked up to 250 ml with distilled water. Aliquots of 2 ml (2 replicates per extract) were placed in a 20 ml Excelo tube and 1 ml of 5% phenol added quickly followed by 5 ml of concentrated sulphuric acid. After shaking the tubes to initiate the reaction, they were then placed in a water bath set at 25°C for 15-30 minutes to allow the colour to stabilise. Absorbance was measured at 490nm using a Pye Unicam SP6-400 UV spectrophotometer. The amount of soluble sugar was determined by reference to a standard curve, prepared from the reaction mixture on a range of D-glucose solutions (2ml aliquots containing 10-100 μg).

2.7 Measurement of fungal growth in compost - 'Race Tube' technique

Using a similar method to that outlined by Tschierpe (1983), triplicate 30g (fresh weight) compost samples were placed in 200 mm glass boiling tubes (30mm diameter) and compacted to a depth of 100 mm after first adding 6 grains of mushroom inoculum (equivalent to 1 per cent inoculation) to the bottom of each the tube. All the tubes were stoppered with cotton wool bungs and incubated at 25°C. From day 7 onwards, and at 7 day intervals up to day 28, mycelial growth fronts were recorded. This was accurately achieved by averaging the growth front measurements at 4 equidistant points around the circumference of the tube. Mycelial advance was represented as mm per day. Fungal contaminants, when observed were identified and noted.

In later investigations, an additional 3 tubes were prepared and inoculated for each compost treatment. These tubes were kept for three weeks before assaying for laccase activity (see 2.11) to assess fungal biomass.

2.8 Measurement of fungal growth in liquid culture

Mushroom mycelium (see Chapter 6) was cultured in two liquid media (a) 2% malt extract and (b) a medium based upon that of Treschow (1944). Both media contained KH_2PO_4 and Na_2HPO_4 salts to maintain a pH close to 7 prior to inoculation (see Appendix 1). Flasks, (normally 250 Erlenmeyer) were prepared containing 50 ml of medium and after inoculation with 2 x 5 mm agar plugs taken from an actively growing mycelial colony, were maintained in a growth cabinet at 25±1°C. After defined periods of time the fungal biomass was collected by vacuum filtration through pre-dried, pre-weighed 0.9 cm Whatman glass microfibre filter papers (Cat. No. 1820 090). The filter papers were dried at 80°C to constant weight to determine accurate weights of fungal biomass. Supernatant solutions were collected and laccase activity (μ moles O_2 min^{-1}) measured using the technique described in 2.11.

2.9 Extraction and analysis of humic substances.

2.9.1 Sonication technique

Freeze dried compost (0.3g) was placed in a 100 ml glass centrifuge tube and 25 ml of 0.5 M NaOH added. (This concentration of alkali was chosen as the majority of published techniques for humic acid extraction from soil samples employ 0.5 M NaOH). The glass tube was firmly clamped and immersed into a beaker of crushed ice to keep the extractant temperature below 10°C. Using a RAPIDIS (180) Ultrasonic Disintegrator generating a high power 20kHz ultrasonic signal, samples of the same compost were sonicated for periods ranging from 2 - 16 minutes using a 18 mm diameter titanium probe tip immersed 10 mm below the extractant surface. At the commencement of each sonication the fitted tuner was adjusted to give maximum audible sound because the cavitation generated can be heard even though the 20kHz waves cannot. Replicate compost samples were taken for each sonication treatment. After completion of each sonication, samples were initially filtered through miracloth (Quick filtration material for gelatinous grindates, CALBIOCHEM, CALIFORNIA) to remove the straw debris (debris 1). This fraction was dried to constant weight at 80°C. The cloudy supernatant was centrifuged (9,000 rev. min^{-1} for 15 min) to give alkali insoluble debris 2. This fraction was removed from the centrifuge tube with the minimum amount of distilled water (2/3 ml) and dried to constant weight in a porcelain crucible. The clear light brown coloured filtrate was then acidified to pH 2.0 using N HCl and left to stand overnight to precipitate humic acid. The alkali soluble/acid insoluble humic material (humic acid) was then separated from the straw coloured filtrate (fulvic fraction) by centrifugation (9,000 rev. min^{-1} for 15 min). The complete procedure is shown in Fig 3. After a number of sonication runs to determine an ideal time for the extraction of humic material it was decided to opt for a 10 minute sonication period for the reasons outlined in Chapter 4.

In order to obtain sufficient levels of both humic and debris 2 fractions to perform nutrient tests on agar (Chapter 6), 0.5 kg of fresh compost (H.R.I. formulation) was divided up into 5 x 100 g samples, each sample being placed in a 1l conical flask with 500 ml of 0.5 N NaOH and shaken gently for 30 min . The samples were individually filtered through miracloth and the supernatants collected, bulked up, and centrifuged at 9,000 rpm to collect the debris 2 fraction (Plate 14) which was later freeze dried. The clear supernatant was acidified with N HCl to pH 2.0 (as outlined above) to precipitate the humic fraction and flasks were allowed to stand overnight. The humic fraction was separated from the clear straw coloured solution (fulvic fraction) by centrifugation at 9,000 rpm and freeze dried.

2.9.2 Analysis of humic and fulvic fractions

In all cases where compost samples were analysed for humic acid content, four replicate samples (0.3 g) were used. Two samples were dried as outlined above and two samples re-acidified (to dissolve the material) and made up to a known volume (100ml) at pH 7.0. Two samples from each replicate extraction were analysed for soluble carbohydrate, phenol, protein and total nitrogen content and the readings averaged.

The clear straw coloured filtrate remaining (fulvic fraction) after debris 1, debris 2 and humic fractions were removed was made up to a known volume (250 ml) and the pH adjusted to 7.0. As above, the fulvic fraction was analysed for soluble carbohydrate, phenol, protein and total N content. As above, two samples from each replicate extraction were analysed and the readings averaged.

(1) Soluble carbohydrate

Soluble sugars within the humic acid complex were measured colorimetrically as described in 2.6 (5) and determined by reference to a standard D-glucose curve.

(2) Phenol content

The total phenol content of the humic acid complex was determined using the Folin-Ciocalteau method (Singleton & Rossi, 1965). To a 1 ml sample of the humic acid fraction dissolved in 0.5 M NaOH, 5 ml of 1/10 aqueous dilution of Folin-Ciocalteau reagent was added. After a minimum of 1/2 min (and before 8 min), 4 ml of Na_2CO_3 were added until a light yellow/green colouration was apparent. Absorbance was measured at 690nm after 1 hour using a Pye Unicam SP6-400 UV Spectrophotometer. The total phenolic component was determined by reference to a standard curve, prepared from the reaction mixture on a range of gallic acid solutions (1 ml volumes containing 10-100 μg of phenol).

(3) Total protein

The total protein content of the humic and fulvic fractions was determined using the COOMASSIE BLUE method (Bradford, 1976). To 1 ml samples, 5 ml of prepared dye reagent (Coomassie Brilliant Blue G-250) was added and thoroughly mixed. The absorbance of the blue colouration that develops after 5 min was read at 595 nm wavelength. A standard curve was made using bovine serum albumin solutions (1 ml containing 6-60 μg protein).

2.10 Assembly of compartmentalised deep column.

To monitor enzyme profiles during cropping and their effect on degradation of the substrate, two identical elongated boxes or columns 1.15m high were constructed out of ply-wood of such internal dimensions (140 mm square) that no overheating of the compost mass would occur once filled. One side panel of each column was subdivided equally into 5 sections (0.23m), each of which could be removed separately during the course of the experiment. These sections were termed, top, upper middle, middle, lower

middle and bottom. Commencing from the base, each section was filled with identical weights (2.3kg) of colonised compost (14 day spawn run) and the operation was repeated for a second column. Three days after filling, one column was 'cased' with a peat/chalk mixture to initiate the fruiting cycle whilst the second column was kept, for the duration of the experiment, as an 'uncased' control. Both columns were placed in a growth room set at 18°C throughout the whole period of the experiment.

At the onset of primordium formation and during periods when mushrooms were developing on the casing soil surface, compost samples (c.50 g) were cleanly cut out from each of the five sections commencing on the left hand side of the column. Further samples, taken at later dates, were then cut to the right of the first sample, such that an uninterupted mycelial network of mycelium, growing vertically downwards from the casing soil surface was sampled each time. This procedure was rigidly followed to overcome any disturbance in enzyme activity that might have been induced by removal of the previous sample. Fresh weight compost samples (one from each section) were extracted as described in 2.9.2 and the supernatant stored in the freezer until required for enzyme analysis (laccase, endocellulase, β-N-acetyl glucosaminidase and β-N-acetyl muramidase). The residual fresh weight of compost remaining from each sample (c.30g) was freeze dried, ball milled and stored for later analysis of debris 1, debris 2, humic and fulvic fractions. On the same day, this procedure of sampling and analysis of compost was repeated on the 'uncased' column.

2.11 Measurement of laccase activity

Mushroom biomass colonising composted substrates was estimated by measuring the activity of the extracellular polyphenol oxidase enzyme, laccase (E.C. 1.14.18.1), produced in large quantities by the mushroom during its vegetative growth phase. The enzyme has been shown to be produced in direct proportion to mushroom mycelium present within a substrate (Wood & Goodenough, 1977). A standard procedure of emptying

the colonised contents from 3 replicate race tubes of each treatment after 21 days and carefully extracting the cereal grain inoculum was adopted. The colonised compost was thoroughly mixed and replicate 20g samples were shaken in 160 ml of distilled water for 1 hour at 25°C. The brown clear supernatant was filtered through nylon gauze to remove the coarse solid debris. The filtrate was further clarified by centrifugation at 12,000 rev. min^{-1} for 15 min. and the brown clear supernatant stored in small polypropylene bottles at -10°C prior to analysis. The addition of an enzyme substrate, *p*-phenylenediamine, to known volumes of extract, initiates a reaction during which oxygen is consumed. The resulting change in oxygen concentration occurring in the sample was measured polarographically using an oxygen electrode, a method outlined by Wood & Goodenough (1977). The unit of laccase activity was defined as the amount of enzyme which consumed 1 μmole of oxygen per minute and values were calculated as enzyme units per ml extract. Mushroom biomass was estimated on the basis that 102 units of enzyme activity is equivalent to one gramme dry weight of mushroom mycelium (correlation, r = 0.944; Wood, 1979).

This method of analysis to assess fungal biomass was also used on the duplicate compost samples taken from deep columns of colonised compost (see Chapter 5).

2.12 Measurement of extracellular endo-cellulase.

The activity of the extracellular enzyme endocellulase in the compost after mushroom inoculation was monitored in the later trials using an improved viscometric method first described by Manning (1981). Duplicate 20g fresh weight samples of colonised compost were taken and immersed in distilled water (160ml) and agitated for 1 hour at 25°C. A clear supernatant was obtained by filtration and centrifugation as outlined in 2.11 and stored at -10°C until required for analysis. Cellulase (carboxymethylcellulase, 1-4-β-D-glucanohydrolase) activity was assayed in an Ubbelohonde viscometer (size

3) maintained at 30 ± 0.05°C in a controlled-temperature water bath and measured in arbitrary units as the change in specific fluidity of the reaction mixture with time ($\eta_{sp}^{-1}s^{-1}$). The reaction mixture, contained in a total volume of 20 ml: consisted of 16 ml 2.5% (w/v) carboxy-methyl cellulose low viscosity solution (BDH Chemicals Ltd), 2 ml 0.2 M sodium acetate/acetic acid buffer (pH 5) with 2 ml of the sample enzyme extract.

The clear supernatants remaining (2 replicates per sample point) were frozen and stored for later analysis of bacteriolytic enzymes.

2.13 Measurement of β-N-acetyl muramidase.

Cell Wall Production: *Bacillus* cell walls were produced by growing *Bacillus subtilis* in batch culture for 3 hours at 37°C in nutrient broth and harvested by centrifugation at 10,000 rpm. Cells were broken down in a Biospec bead beater, and boiled in an equal amount of 4% SDS. On cooling, the cell walls were washed twice by centrifuging and resuspended in distilled water. A stock solution was prepared by suspending *Bacillus* cells in 0.05M acetate buffer at pH 5 so there was 600μg dry weight in 310μl buffer. This produced an optical density of 0.45 at 600nm in a 500μl cuvette (masked sides) with a 10mm path length.

The activity of the wall degrading enzyme β-N-acetyl muramidase was assayed by measuring the rate of decrease of optical density ($\Delta OD_{600}h^{-1}$) of the stock mixture containing *B. subtilis* walls together with 100 μl of test supernatant (2 replicates) when incubated at 25°C for 30 min (Grant *et al*, 1986). A unit of activity was defined as the quantity of enzyme in 100μl of supernatant catalysing a ΔOD_{600} of 0.001 in 1 min under the assay conditions outlined.

2.14 Measurement of β-N-acetylglucosaminidase (E.C. 3.2.1 30).

Culture supernatant (0.2 ml) was added to a solution (0.4 ml) of *p*-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma; 1 mg ml^{-1}) in 0.05 M acetate

buffer pH 5.0 and incubated at 25°C for 1 hour. The reaction was terminated by the addition of 1 M Na_2CO_3 (2 ml). The released *p*-nitrophenol was measured spectrophotometrically at 400nm and enzyme activity was calculated as μ mol *p*-nitrophenol released min^{-1} [ml culture supernatant] (Fermor & Wood, 1981). As above, this assay was performed on supernatants obtained from 2 separate compost samples.

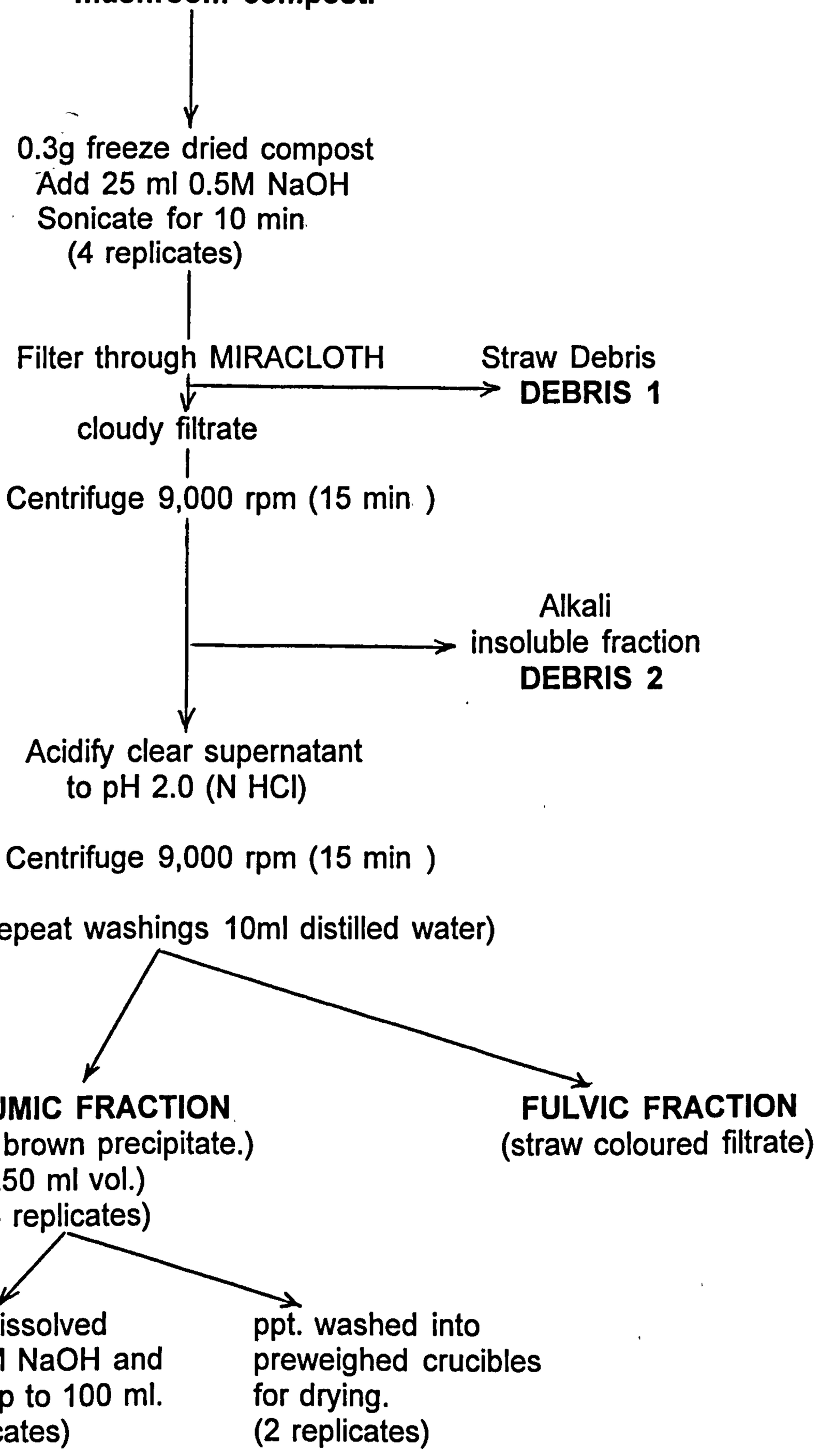
2.14 Determination of proteolytic activity.

Proteolytic activity was assayed spectrophotometrically (Burton *et al.*, 1993) by following the release of *p*-nitroaniline from succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide (Suc-Ala-Ala-Pro-Phe-*p*NA). The assay mixture contained 0.15 mM-Suc-Ala-Ala-Pro-Phe-*p*NA in 100 mM-sodium phosphate buffer, pH 7.5. Enzyme activity was measured by following the initial increase in A_{405} at 35°C (Burton *et al.*, 1993). A unit of activity was defined as the amount causing an increase of 1 absorbance unit min^{-1}.

2.15 Statistical Analysis

Wherever possible (i.e. adequate replication), the data was analysed statistically (ANOVAR) and the least significant differences represented at the 5% level.

Figure 3. Flowchart of extraction of humic and fulvic acids from mushroom compost.



CHAPTER 3 MYCELIAL COLONISATION RATE OF COMPOSTED SUBSTRATES AS A MEASUREMENT OF COMPOST SELECTIVITY

3.1 Procedure

As outlined in 2.3 and 2.4, a traditionally prepared mushroom compost was taken and subjected to prolonged composting periods at 45°C and 55°C for periods of 1, 2 and 3 weeks to produce a range of degraded substrates for testing. On completion of set composting periods, three 'race tubes', each containing 30 g of prepared compost were set up for each *Agaricus* strain tested and the mycelial growth rate at 25°C from the inoculation point was measured (mm/day) over a 28 day period (See 2.7).

3.2 Experiments 1 & 2.

In experiments 1 and 2, the waterbath temperature of both composting units was set at 45°C, a temperature favourable for the development of thermotolerant fungi. Two flasks were selected at random (one from each composting unit) after 1, 2 and 3 weeks and the contents pooled and well mixed before sampling for analysis. In both experiments thermotolerant fungi were clearly visible on the compost surface of flasks receiving a prolonged composting period of 1 week. It was also noticeable that these fungi were more apparent in composts receiving 2 and 3 weeks extended composting and microscopic examination of the substrate confirmed the presence of this group of organisms. Thermophilic actinomycetes were also apparent as sporulating colonies on the straw surface of composts receiving 1 weeks additional composting, although this condition became less apparent when composting was prolonged for 2 and 3 weeks.

Analysis of composts from both experiments (Table 3) demonstrated that compost dry matter losses were around 8% within the first week and between 14-19% after three weeks. As the compost ingredients had initially undergone the traditional two phase procedure before filling into flasks, very little nitrogen was evolved as ammonia during the extended composting periods. Consequently the loss of carbon to the atmosphere as CO_2 was far greater than any loss of nitrogen as ammonia and this was reflected by the steady rise in total nitrogen in both experiments. It was also apparent that moisture content of the composts in both experiments rose steadily to the extent that the 3 week compost had a moisture content approximately 3% greater than the control compost. In both experiments, 7 *Agaricus* species were selected for testing on the range of substrates produced. The only difference between experiments was that in Expt:2 a different strain of *Agaricus bisporus* was used.

The growth rate of mushroom mycelium through the compost was significantly affected by the extended periods of composting as shown by the race tube analysis outlined in Table 4, and Figures 4-7. In both experiments significant improvement in growth rate above the control compost was apparent for all species except for *A. campestris* (the field mushroom) when composting was prolonged for 1, 2 and 3 weeks at 45°C. As no growth whatsoever was recorded for *A. campestris* on any of the substrates produced in Expts 1 & 2 and spawn preparation on grain was unsuccessful, this species was omitted from further studies.

The interpretation of the above results at this early stage of the investigation remained unclear as there were many variables as shown by the compost analyses that could influence colonisation rate. Nevertheless, there was clear evidence that the rate of compost colonisation by mycelium could be improved by extending the composting period at 45°C and that the major improvement was achieved after 7 or 14 days.

Table 3. Changes of composition in a traditionally prepared mushroom compost when subjected to further composting periods of 1, 2 and 3 weeks at 45°C.

Experiment 1

Treatment	Weight (g)	% H_2O	Total dry matter (g)	% Loss dry matter	% ash	Total (N) % dry matter	pH
Control	800.0	71.8	225.6	0	15.99	2.00	7.5
+ 1 week	770.2	72.9	208.7	7.49	20.43	2.10	7.7
+ 2 weeks	771.5	74.1	199.8	11.43	18.96	2.20	7.7
+ 3 weeks	759.6	74.6	192.9	14.49	21.51	2.40	7.4

Experiment 2

Treatment	Weight (g)	% H_20	Total dry matter (g)	% loss dry matter	% ash	Total (N) % dry matter	pH
Control	800.0	70.0	240.0	0	23.11	1.98	7.65
+ 1 week	769.9	71.3	220.9	7.95	23.75	2.46	7.2
+ 2 weeks	750.5	73.0	202.6	15.58	29.99	2.72	7.45
+ 3 weeks	710.5	72.6	194.7	18.87	27.69	2.80	7.2

Table 4. Mycelial advance (mm/day in 'Race tubes') of 7 *Agaricus* strains in a traditionally prepared compost that had received 1, 2 and 3 weeks prolonged composting at 45°C prior to inoculation.

Experiment 1.

Species	Control	+1 week	+2 weeks	+3 weeks	Lsd (5%)
A. bisporus (D649)	3.11	6.23	7.14*	7.14*	0.69
A. bitorquis (DDR 30/4)	1.35	4.59	5.33	4.38	1.48
A. bitorquis (DDR 36/2)	1.47	4.69	5.57	6.16	1.05
A. bitorquis (W20)	0.57	3.80	2.42	4.04	0.97
A. brozei (W28)	4.47	7.14*	7.14*	7.14*	0.69
A. macrosporus (W7)	0.71	2.69	2.50	2.28	1.00
A. campestris (W1A)	NG	NG	NG	NG	

Experiment 2.

Species	Control	+1 week	+2 weeks	+3 weeks	Lsd (5%)
A. bisporus (U1)	0.98	5.57	5.97	5.30	0.73
A. bitorquis (30/4)	0.69	4.17	4.42	2.83	0.46
A. bitorquis (36/2)	0.71	3.69	4.80	3.73	0.89
A. bitorquis (W20)	0.64	3.33	3.93	3.07	1.52
A. brozei (W28)	2.80	6.14	4.54	6.26	1.36
A. macrosporus (W7)	NG	1.19	0.92	0.78	0.49
A. campestris (W1A)	NG	NG	NG	NG	

Mycelial advance (mm/day) measured after 14 days growth
(6 replicate tubes per treatment).

NG = No growth recorded
* = Whole column colonised (i.e. 100 mms, at or before day 14.)

Figure 4. Race-tube mycelial growth rates of Agaricus spp. (Expt.1)
on substrates receiving prolonged composting periods at 45°C

Experiment 1.

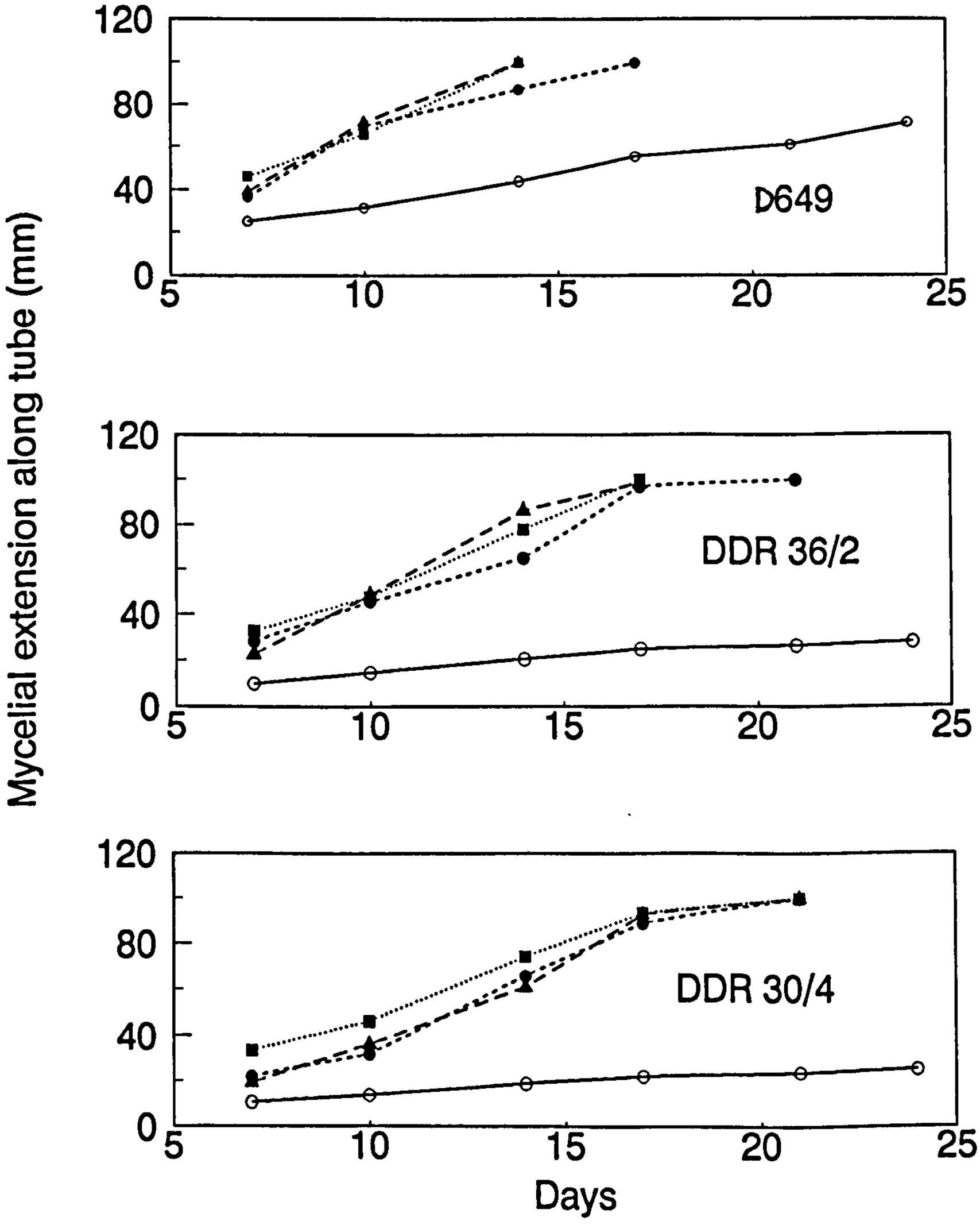


Figure 5. Race-tube mycelial growth rates of Agaricus spp. (Expt.1)
on substrates receiving prolonged composting periods at 45°C

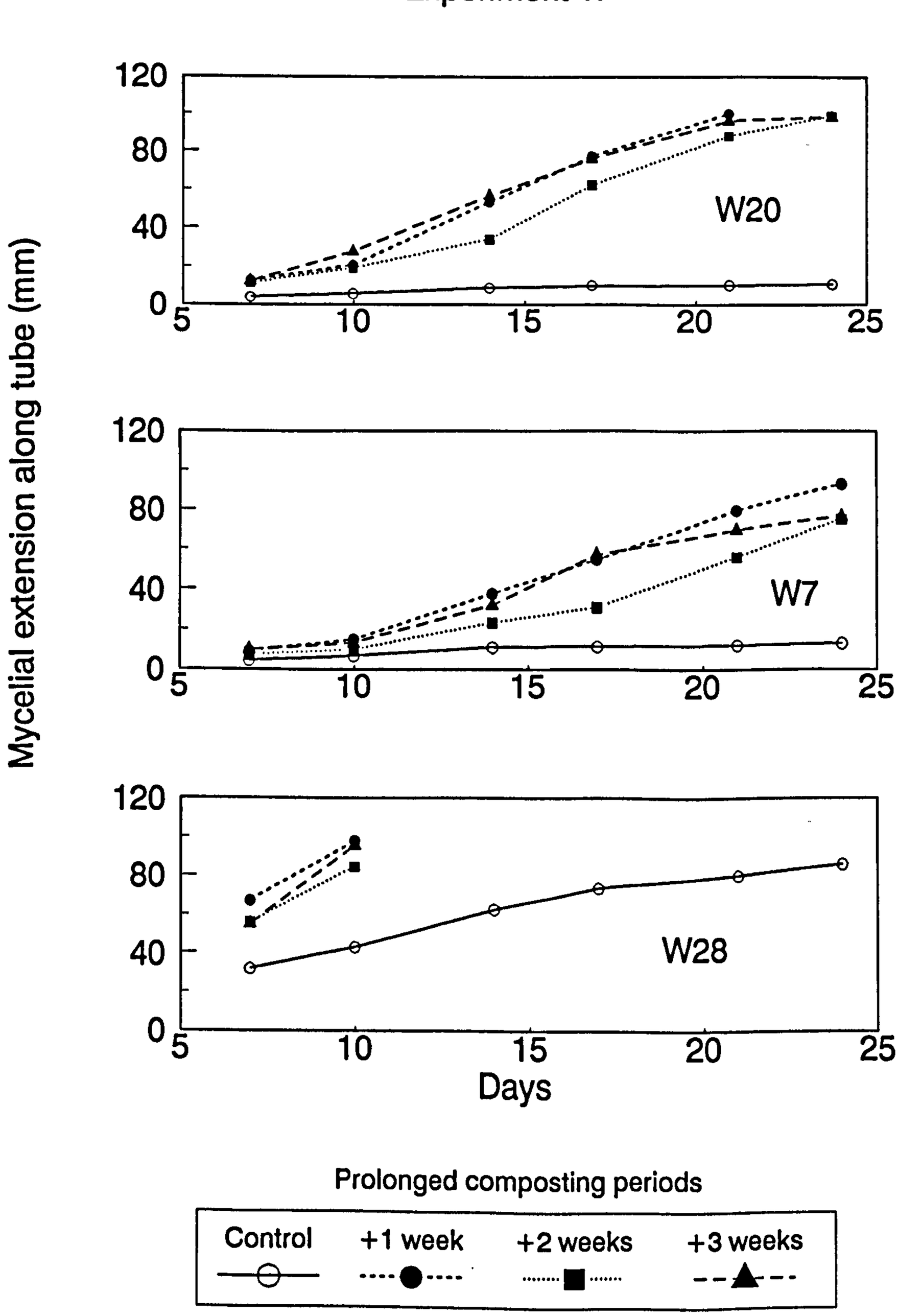


Figure 6. Race-tube mycelial growth rates of Agaricus spp. (Expt.2)
on substrates receiving prolonged composting periods at 45°C

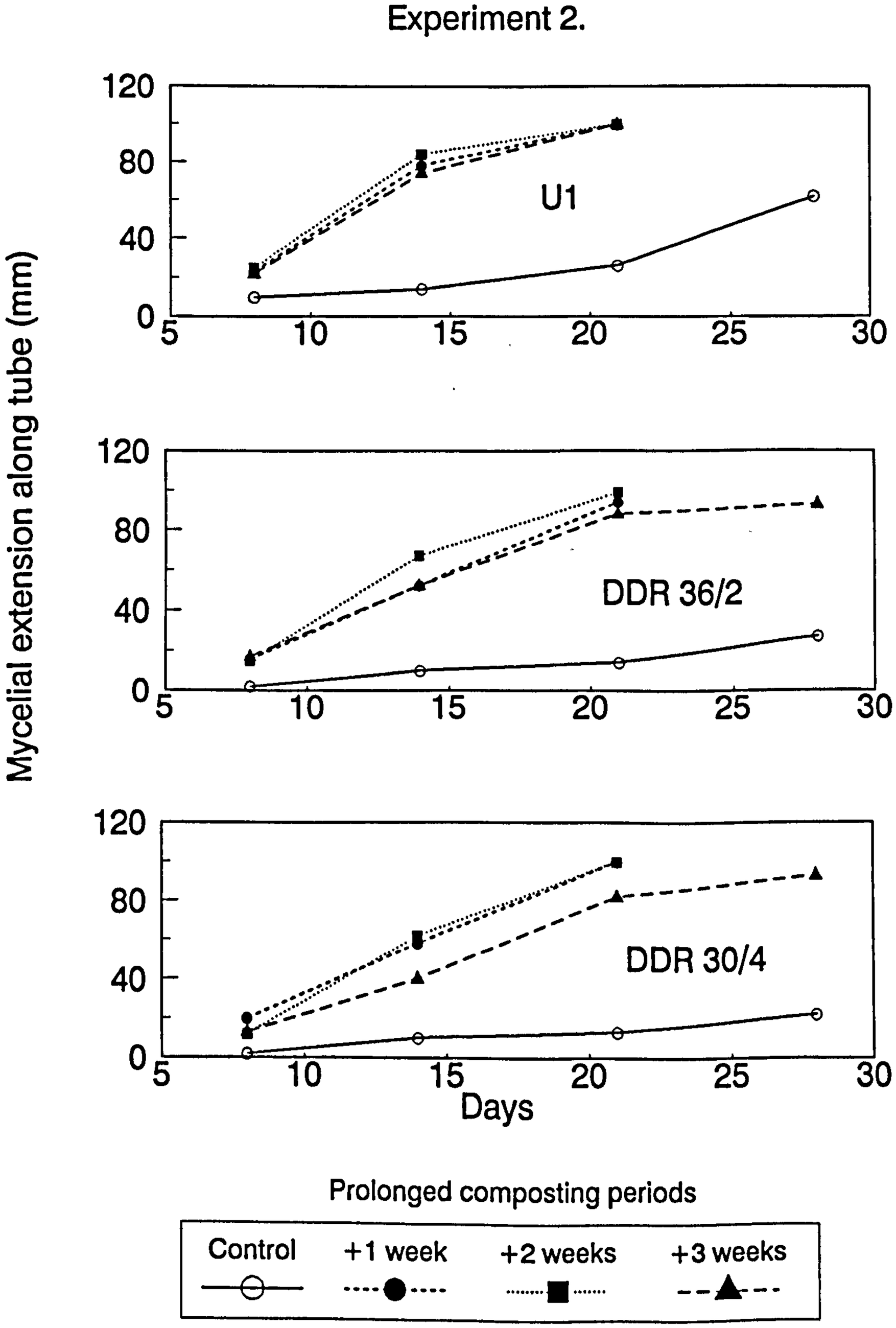
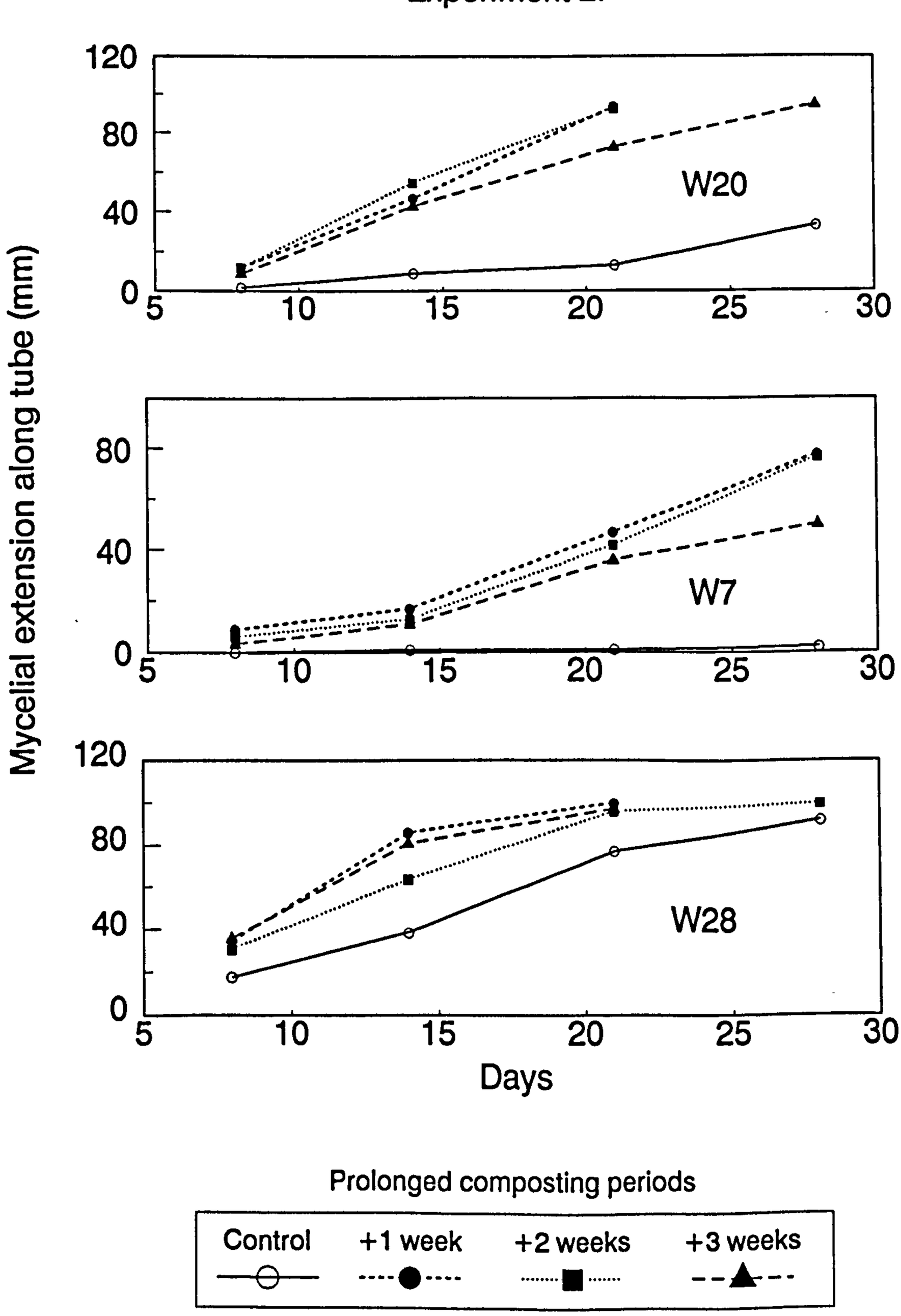


Figure 7. Race-tube mycelial growth rates of Agaricus spp. (Expt.2)
on substrates receiving prolonged composting periods at 45°C



3.3 Experiment 3

Experiment 3 was designed to investigate whether the improvements in mycelial growth rate recorded in the previous experiments would occur when a traditional mushroom substrate was subjected to prolonged composting periods of 55°C. This temperature would be more favourable for thermophilic bacteria and actinomycete development. To make this comparison, the waterbath of one composting unit was maintained at 45°C while the other was maintained at 55°C. As with the previous experiment, flasks were selected at random after 1, 2 and 3 weeks (n.b. only 1 flask per treatment in this experiment). As with Experiments 1 & 2, the flask contents were accurately weighed after each composting period and samples freeze dried for later analysis. Six *Agaricus* strains were once again selected for testing on the 7 compost types (i.e. Control compost together with 6 compost treatments). In this experiment the amount of dry matter loss with time was much less than with Expts. 1 and 2, the compost receiving an extended period at 55°C giving the greatest dry matter loss after 3 weeks of about 10% (Table 5).

Agaricus strains that were grown on composts that were prepared by extending the composting time at 45°C once again gave significant improvements in colonisation rate for 5 of the 6 species tested (Table 6), even though the colonisation rate for the control treatments were better than in the previous two experiments. The same *Agaricus* strains when grown on composts that were prolonged at 55°C for 1 and 2 weeks also gave significant improvements in their colonisation rate but a significant decline in colonisation rate were recorded for composts that had received a composting duration of 3 weeks at this temperature.

3.4 Experiments 4 and 5.

As described in 2.3, glucose was added to the starting composts of Expts 1, 2 and 3 (at approximately 0.9 g/100 g fresh weight compost) to increase the soluble carbohydrate level of the control compost to around 3 per cent of the compost dry matter. This addition was to ensure that the resident micro-organism population established after two phases of composting had a readily available carbon source present at the recommencement of a forced composting regime and as discussed in 2.3, to discourage ammonia production. Experiments 4 and 5 conducted at 45°C and 55°C respectively (with and without glucose supplementation) were designed to answer the question whether observations made for Expts. 1 to 3 were genuine and not in any way related to an improvement in microbial biomass due to carbohydrate supplementation.

Changes in composition for composts produced at 45°C and 55°C, with and without glucose supplementation were very similar (Tables 7 & 8). Dry matter losses after 3 weeks additional composting were between 16-20% and it was also apparent, as with the earlier experiments, that the moisture content increased quite markedly with the duration of composting. Soluble carbohydrate analysis of composts before and after supplementation with glucose are shown in Figure 8. At the re-commencement of composting, the soluble carbohydrate level of the control compost was increased from around 0.7% to 2.9% and 2.6% of the dry matter for Expts. 4 and 5 respectively. As shown in Figure 8, after 7 days further composting there was no significant difference in totally available soluble carbohydrate remaining irrespective of composting regime, and the level of soluble carbohydrate extracted from the compost was at a level expected of a selective mushroom compost at the time of spawning i.e below or around 1% of the dry matter (Smith & Spencer, 1977). This was also true for composts receiving 2 and 3 weeks additional composting. While glucose had been added to earlier compost trials to suppress ammonia production, the unsupplemented controls were all ammonia-free at the time of spawning, no

Table 5. **Analysis of changes occurring in a traditionally prepared mushroom compost when subjected to further composting periods of 1, 2 and 3 weeks at 45°C and 55°C.**

Experiment 3 (45°C)

Treatment	Weight (g)	% H_20	Total dry matter (g)	% loss dry matter	% ash	Total (N) % dry matter	pH
Control	800.0	72.0	224.2	0	16.37	1.80	7.75
+ 1 week	770.8	71.1	222.8	0.62	18.84	2.10	7.80
+ 2 weeks	779.7	72.2	216.7	3.34	19.24	2.06	7.65
+ 3 weeks	762.5	72.4	210.4	6.15	16.14	2.09	7.85

Experiment 3 (55°C).

Treatment	Weight (g)	% H_20	Total dry matter (g)	% loss dry matter	% ash	Total (N) % dry matter	pH
Control	800.0	72.0	224.2	0	16.37	1.80	7.75
+ 1 week	765.0	71.1	221.2	1.33	16.59	2.17	7.95
+ 2 weeks	764.5	72.3	211.8	5.36	17.09	2.08	7.52
+ 3 weeks	749.9	73.0	202.4	9.72	23.76	2.49	7.80

Table 6. **Mycelial advance (mm/day in 'Race-tubes') of 6 *Agaricus* strains in a traditionally prepared compost that had received 1, 2 and 3 weeks prolonged at 45°C and 55°C prior to inoculation**

Experiment 3 (45°C).

Species	Control	+1 week	+2 weeks	+3 weeks	Lsd (5%)
A. bisporus (D649)	3.42	6.04	6.19	6.50	0.80
A. bitorquis (K32)	5.42	6.14	3.50	4.64	1.71
A. bitorquis (W19)	4.33	5.73	2.85	6.21	1.11
A. bitorquis (W20)	4.02	5.78	2.61	5.21	1.83
A. silvaticus (W4II)	2.07	2.66	2.23	3.64	0.42
A. nivescens (WH23)	2.21	3.07	3.09	3.07	1.19

Experiment 3 (55°C).

Species	Control	+1 week	+2 weeks	+3 weeks.	Lsd (5%)
A. bisporus (D649)	3.42	6.42	5.52	3.09	1.99
A. bitorquis (K32)	5.42	6.02	5.95	1.92	0.95
A. bitorquis (W19)	4.33	5.92	6.11	3.52	2.14
A. bitorquis (W20)	4.02	5.23	6.11	2.54	1.13
A. silvaticus (W4II)	2.07	2.73	2.78	1.21	0.41
A. nivescens (WH23)	2.21	3.11	2.90	1.45	0.86

Mycelial advance (mm/day) measured after 14 days growth (3 replicate tubes per treatment).

Table 7. Analysis of changes occurring within composts during prolonged composting periods at 45°C with/without glucose supplementation (Experiment 4).

Treatment	Weight (g)	% H_2O	Total dry matter (g)	% loss dry matter	% ash	Total (N) % dry matter	pH
	(a) without glucose supplementation						
Control	800.0	68.1	255.2	0	12.65	1.84	7.40
+ 1 week	785.2	70.0	235.6	7.68	13.84	2.00	7.42
+ 2 weeks	774.7	70.6	227.7	10.77	14.80	2.25	7.37
+ 3 weeks	759.5	72.2	211.1	17.28	14.24	2.42	7.47
Treatment	(b) with glucose supplementation						
Control	800.0	67.4	260.8	0	12.85	1.81	7.40
+ 1 week	779.6	70.3	231.5	11.23	14.08	2.20	7.44
+ 2 weeks	765.1	71.9	215.0	17.56	14.40	2.53	7.41
+ 3 weeks	765.5	72.6	209.7	19.59	16.00	2.79	7.54

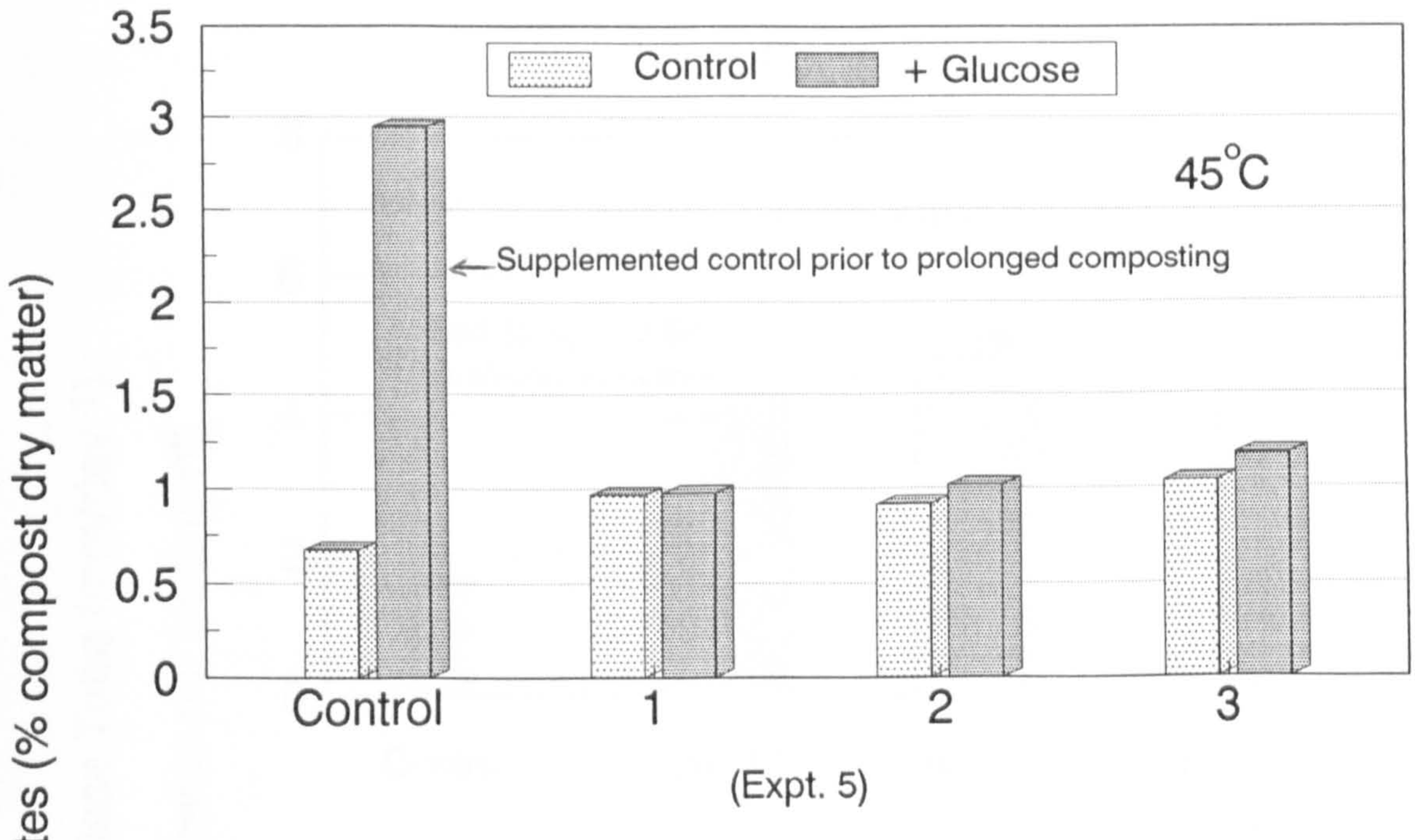
Table 8. Analysis of changes occurring within composts during prolonged composting periods at 55°C with/without glucose supplementation (Experiment 5).

Treatment	Weight (g)	% H_20	Total dry matter (g)	% loss dry matter	% ash	Total (N) % dry matter	pH
	(a) without glucose supplementation						
Control	800.0	75.6	195.2	0	14.32	2.10	7.23
+ 1 week	776.2	77.1	177.7	8.96	17.04	2.45	6.99
+ 2 weeks	769.8	78.4	166.3	14.80	16.28	2.78	7.05
+ 3 weeks	770.6	79.5	160.6	17.72	17.20	2.53	7.08
Treatment	(b) with glucose supplementation						
Control	800.0	74.8	201.6	0	14.20	2.19	7.23
+ 1 week	777.4	74.9	195.1	3.22	17.48	2.75	7.09
+ 2 weeks	775.2	76.7	180.6	10.41	18.40	2.42	7.12
+ 3 weeks	772.1	78.0	169.8	15.77	16.04	2.64	7.13

Figure 8. **Soluble carbohydrate analyses of composts that had received prolonged composting times of 1, 2 and 3 weeks at 45°C and 55°C.**

(with and without glucose supplementation)

(Expt. 4)



(Expt. 5)

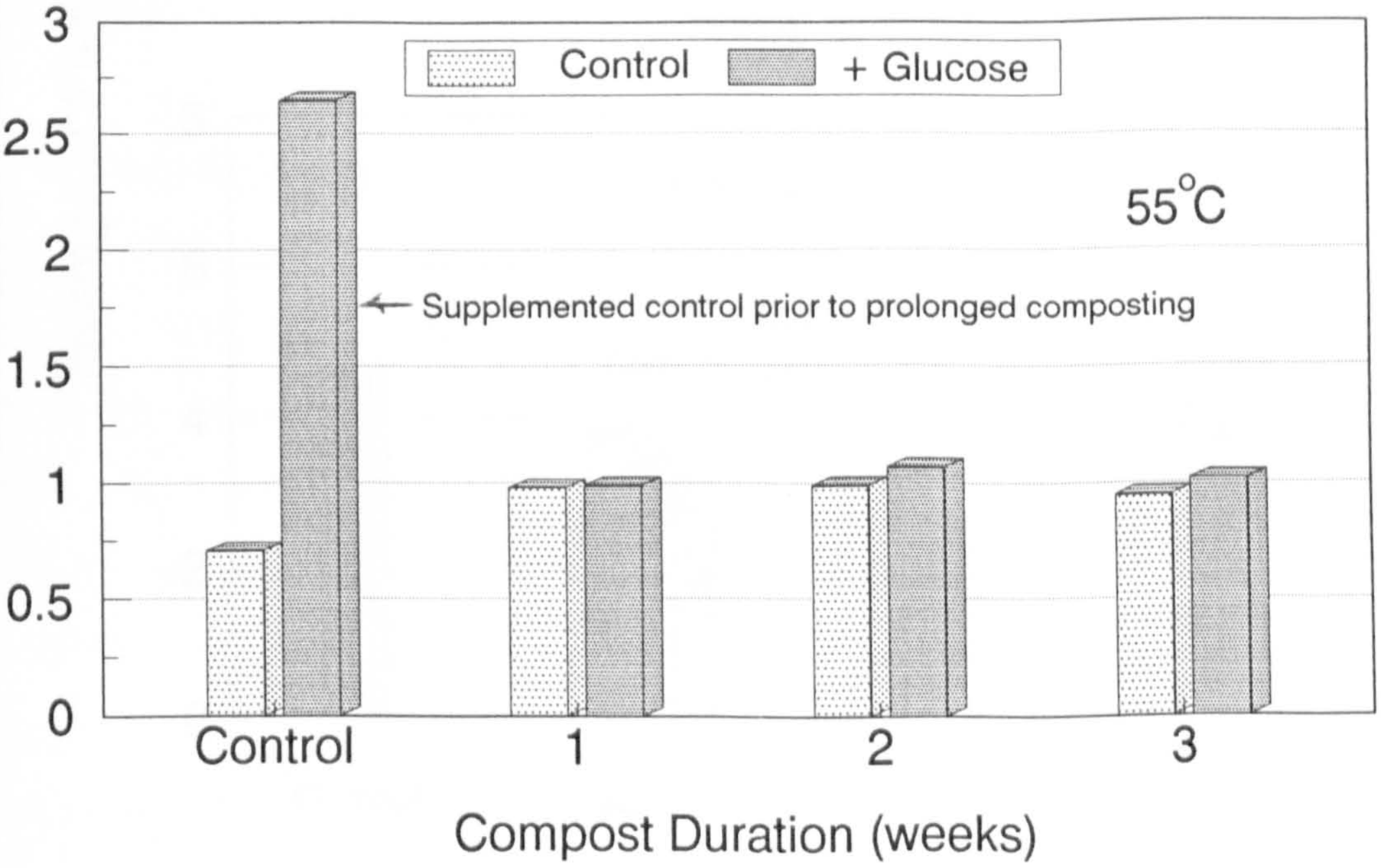
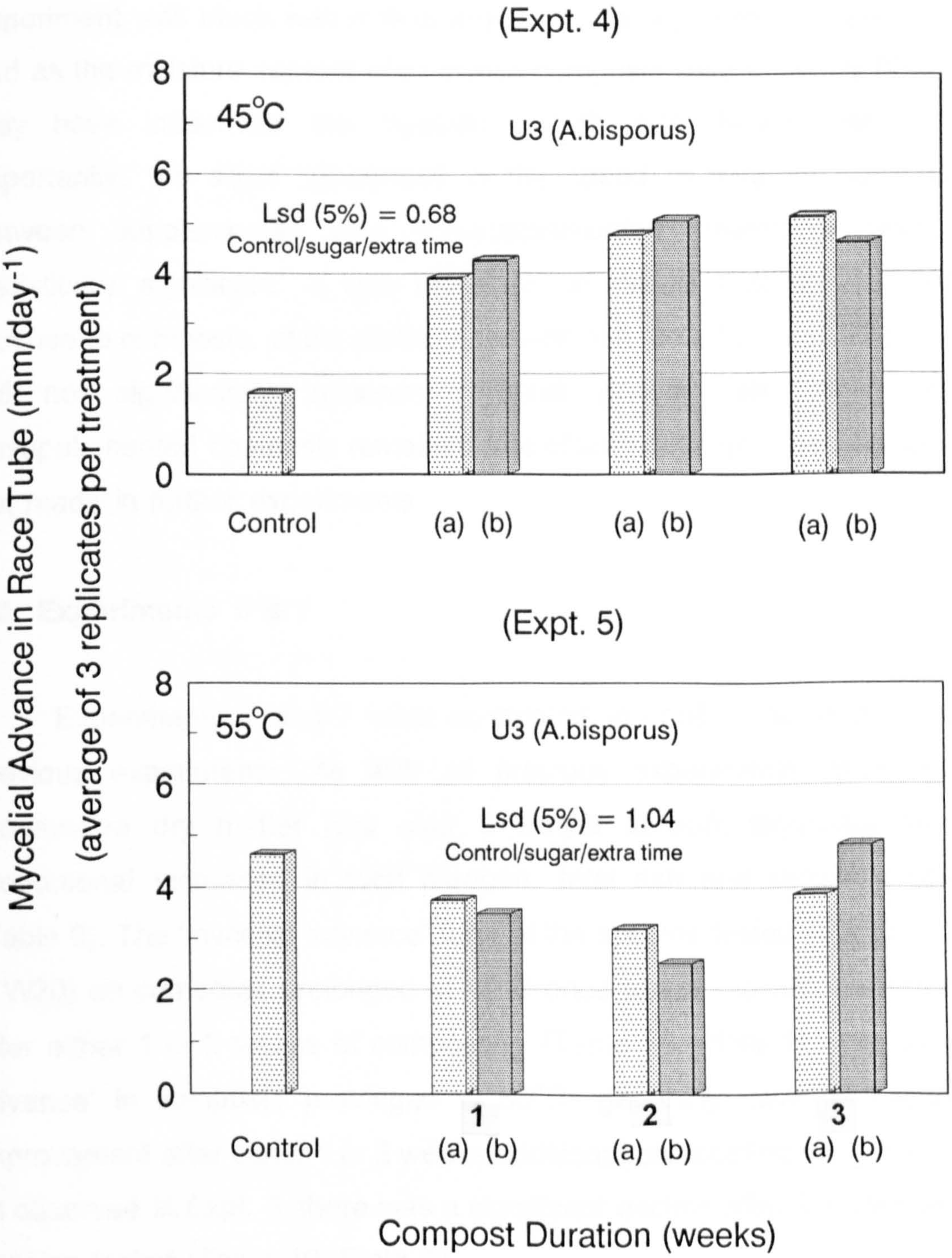


Figure 9. Comparison of mycelial growth rates in composts receiving additional composting times of 1, 2 and 3 weeks at 45°C and 55°C

(a) without (b) with glucose supplementation



matter what temperature or duration were imposed on the compost.

The speed of mycelial colonisation, as measured using the'race-tube' technique, also gave no significant differences between supplemented or unsupplemented composts (Figure 9). The improvement in colonisation rate with prolonged composting at 45°C was once again apparent in Expt. 4. While the observations made for Expt. 5, performed at 55°C, did not conform to the pattern observed for Expt. 3, the control compost used in this experiment was much wetter than any other starting compost used before, and as the moisture content of prepared composts rose to nearly 80%, this may have influenced the mycelial growth rate. Nevertheless, more importantly, the slight differences in the speed of mycelial colonisation between supplemented and non-supplemented treatments were not statistically significant. It was therefore concluded that the addition of glucose to composts, at the commencement of a forced composting regime, did not significantly influence mycelial growth rate. Also, as all unsupplemented composts remained free of ammonia, glucose addition was not made in further experiments.

3.5 Experiments 6 & 7.

Experiments 6 and 7 were conducted to confirm the findings of all previous experiments. As with all previous experiments there was a progressive dry matter loss over 3 weeks at both temperatures with proportional increases in total nitrogen, total ash and moisture content (Table 9). The 'mycelial advance' for 4 of the species tested (U3, W4II, K26 & W20) on composts prolonged at 45°C once again showed improvement after either 1 or 2 weeks of composting (Table 10, Plate 1). The 'mycelial advance' in composts prolonged at 55°C generally gave no significant improvement after either 1 or 2 weeks additional composting but once again, as observed in Expt. 3, there was a significant decline after 3 weeks with all species tested (Table 10, Plate 2).

Table 9. Analysis of changes occurring within composts during prolonged composting periods at 45°C (Expt.6) and 55°C (Expt.7) with/without glucose supplementation.

Treatment	Weight (g)	% H_20	Total dry matter (g)	% loss dry matter	% ash	Total (N) % dry matter	pH
	45°C						
Control	800.0	74.9	200.80	0	14.68	1.92	7.45
+ 1 week	787.9	75.3	194.61	3.08	14.64	2.06	7.58
+ 2 weeks	781.6	76.9	180.54	10.08	16.44	2.34	8.00
+ 3 weeks	765.1	78.2	166.79	16.93	18.64	2.44	8.05
Treatment	55°C						
Control	800.0	76.4	188.80	0	17.76	2.32	7.70
+ 1 week	782.9	77.4	176.93	6.28	21.98	3.00	7.35
+ 2 weeks	790.1	78.9	166.71	11.70	25.46	3.24	8.35
+ 3 weeks	723.7	79.6	147.63	21.80	26.94	3.34	7.75

Table 10. Mycelial advance (mm/day in'race-tubes') of *Agaricus* spp. in a traditionally prepared compost that had received 1, 2 and 3 weeks prolonged composting at 45°C (Expt.6) and 55°C (Expt.7) prior to inoculation.

Species	Prolonged composting at 45°C				
	Control	+1 week	+2 weeks	+3 weeks	Lsd (5%)
A. bisporus (U3)	5.00	5.44	6.34	6.08	0.33
A. silvaticus (W4II)	0.26	1.39	-	-	0.17
A. arvensis (DDR 30/4)	4.23	3.30	4.09	3.85	0.38
A. brozei (W28)	7.09	7.07	7.09	7.10	0.14
A. bitorquis (K26)	4.52	4.33	5.66	4.90	0.80
A. bitorquis (W20)	3.90	3.45	5.44	6.21	0.88
Species	Prolonged composting at 55°C				
	Control	+1 week	+2 weeks	+3 weeks	Lsd (5%)
A. bisporus (U3)	5.96	6.65	6.53	3.25	0.20
A. silvaticus (W4II)	3.13	2.77	1.98	0.92	0.31
A. arvensis (DDR 30/4)	5.03	4.42	5.O3	1.97	0.27
A. brozei (W28)	7.14	7.14	7.14	4.70	0.15
A. bitorquis (K26)	5.44	5.13	5.48	2.48	0.48
A. bitorquis (W20)	4.78	4.67	6.76	3.40	0.59

Mycelial extension rates (mm/day) measured after 14 days growth (6 replicate tubes per treatment).

Table 11. **Laccase activity of 5 *Agaricus* spp. within colonised compost (μ moles O_2/g dry matter) assayed 21 days after mycelial inoculation (2 replicates/treatment).**

(Experiment 6 - 45°C; Experiment 7 - 55°C)

Species	Prolonged composting at 45°C				
	Control	+1 week	+2 weeks	+3 weeks	Lsd (5%)
A. bisporus (U3)	2.66	3.64	4.46	4.39	0.91
A. silvaticus (W4II)	-	0.05	-	-	0.02
A. arvensis (DDR 30/4)	0.32	0.31	0.60	0.47	0.20
A. brozei (W28)	1.51	1.58	2.11	2.03	0.32
A. bitorquis (K26)	0.68	0.56	0.83	0.98	0.64
A. bitorquis (W20)	1.17	1.28	2.15	2.76	0.71
Species	Prolonged composting at 55°C				
	Control	+1 week	+2 weeks	+3 weeks	Lsd (5%)
A. bisporus (U3)	2.17	2.03	2.30	1.15	0.40
A. silvaticus (W4II)	0.79	0.46	0.78	0.18	0.43
A. arvensis (DDR 30/4)	0.65	0.34	0.60	0.06	0.09
A. brozei (W28)	1.03	0.68	1.18	0.54	0.10
A. bitorquis (K26)	0.60	0.39	0.69	0.11	0.27
A. bitorquis (W20)	1.70	1.66	2.48	1.70	0.79

Plate 1. Comparison of mycelial growth of 5 Agaricus spp. after 21 days in composts that had received prolonged composting times at 45°C for 1, 2 and 3 weeks (Experiment 6).

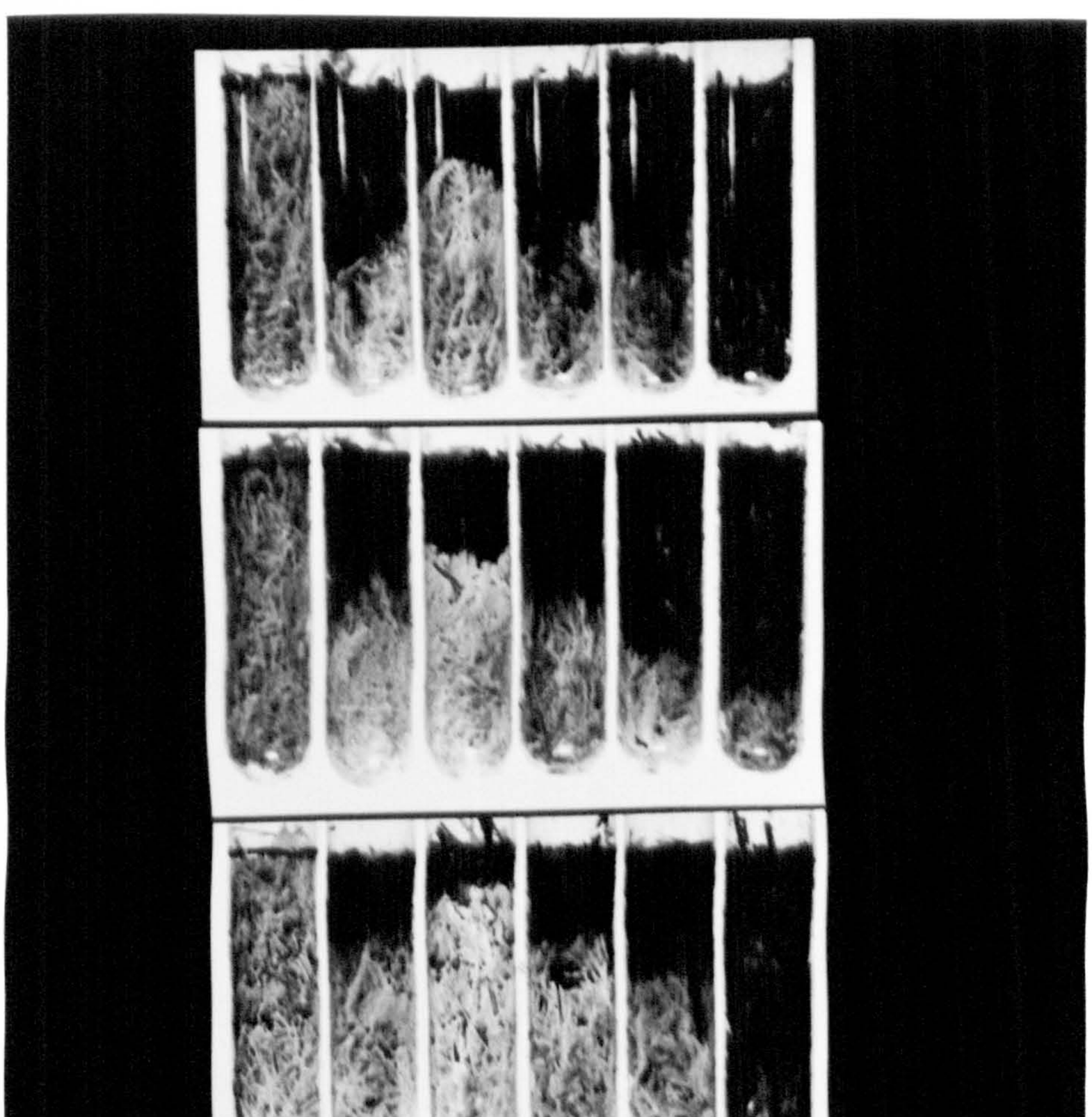
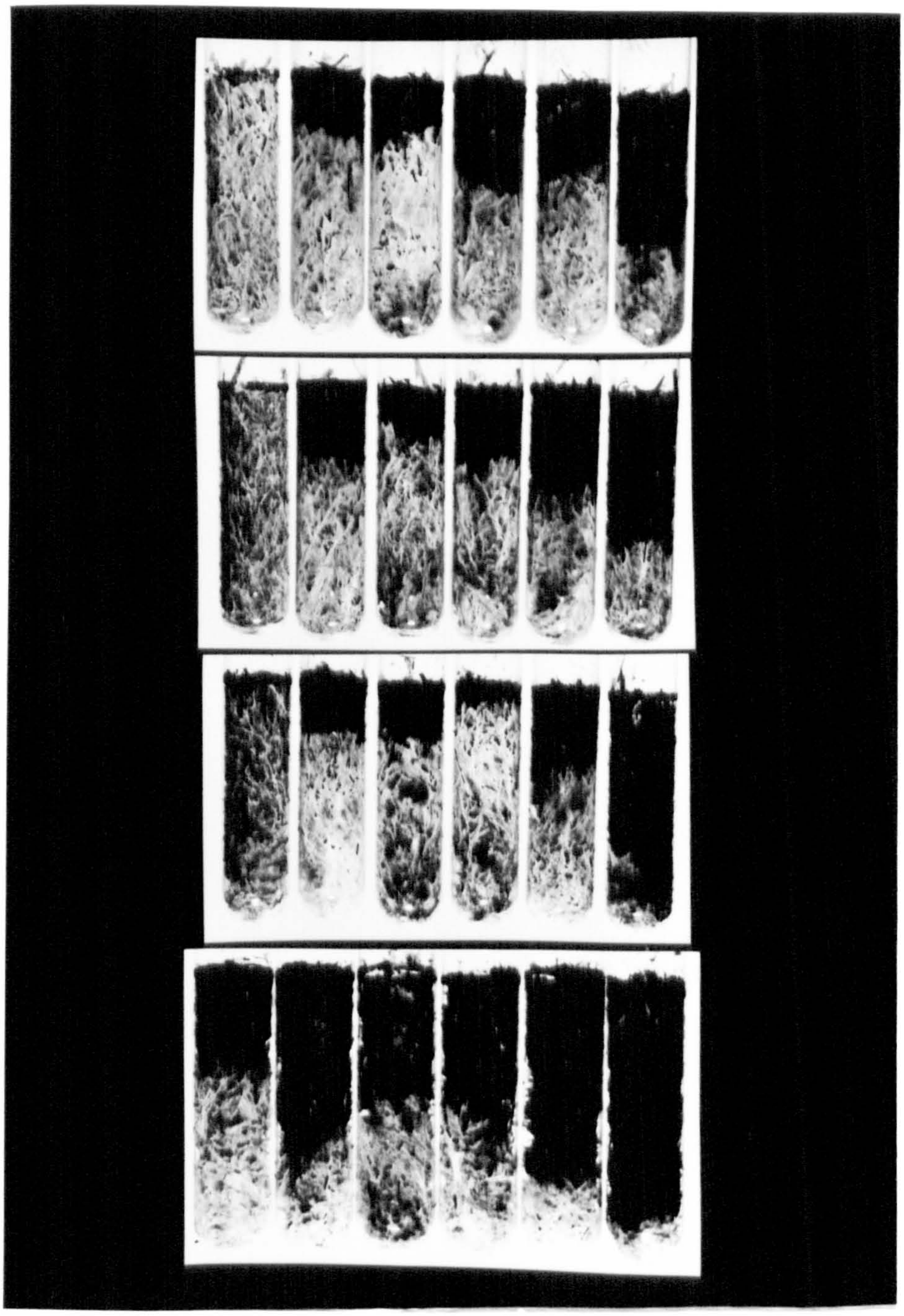


Plate 2. Comparison of mycelial growth of 5 Agaricus spp. after 21 days in composts that had received prolonged composting times of 1, 2 and 3 weeks at 55°C (Experiment 7).



3.6 Experiment 8

In all the previous experiments (i.e. 1 to 7), observations on the colonisation rate of mushroom mycelium had been recorded for a number of strains of commercial as well as wild *Agaricus* species. While it was interesting to compare the mycelial growth rate of a number of closely related species on a wide range of degraded substrates, the preparation of grain inoculum for all these strains generated a considerable work load. As similar observations were made for the majority of the species tested, from Experiment 8 onwards, the nutritional studies relating to compost selectivity were conducted using the more important commercial strains of one species, *Agaricus bisporus*.

Experiment 8 was conducted once again at two temperatures, 45°C and 55°C. The analytical data for both compost treatments are shown in Table 12 In this experiment, an additional treatment was included i.e. compost sterilised by autoclaving (121° C, 15 psi for 1 hour), to determine whether mycelial growth differences recorded in previous experiments would occur in composts where the microflora were killed. As it was more convenient to maintain sterility by inoculating compost in replicate 'race tubes' from the top rather than the bottom, all other unsterile treatments included in this experiment were inoculated from the top. Growth fronts were then measured downwards rather than upwards (see Plate 3). In this experiment an additional 3 race tubes per treatment were also set up for extracellular laccase assay which was measured in colonised compost 3 weeks after inoculation to determine fungal biomass accumulation (Wood, 1979). As with previous experiments the 'mycelial advance' in race tubes was improved significantly in composts receiving an extended composting time of 45°C for 1, 2 and 3 weeks (Figure 10). Although the data for the two prolonged temperature treatments are presented separately in Figures 10 and 11, analysis of variance was performed on all 7 treatments (i.e. control, together with prolonged composting periods of 1, 2, and 3 weeks at either 45°C or 55°C). Consequently the LSD's presented in both figures are common. This is also the case for the autoclaved series presented in Figures 12 and 13.

Table 12. Analytical changes occurring within composts during prolonged composting periods at 45°C and 55°C (Experiment 8). (Only *A.bisporus* [U3] was used).

Treatment	Weight (g)	% H_20	Total dry matter (g)	% loss dry matter	% ash	Total N % dry matter	pH
	Prolonged composting at 45°C						
Control	800.0	75.53	195.76	-	16.34	2.25	7.92
+1 week	777.3	75.77	188.33	3.79	19.02	2.84	7.80
+2 weeks	738.7	76.83	171.15	12.57	19.98	3.05	8.02
+3 weeks	770.8	79.03	161.63	17.43	21.42	3.06	7.98
Treatment	Prolonged composting at 55°C						
Control	800.0	75.53	195.76	-	16.34	2.25	7.92
+1 week	772.4	77.02	177.49	9.33	20.06	2.92	7.78
+2 weeks	763.2	79.02	160.11	18.21	20.66	3.40	8.06
+3 weeks	758.9	78.74	161.34	17.58	24.34	3.49	7.90

Mycelial advance (mm/day in 'Race tubes') of *A. bisporus* in composts that received prolonged composting times at 45°C and 55°C. (Auto - composts autoclaved before inoculation)

Composting temperature	Control	+1 week	+2 weeks	+3 weeks	Lsd (5%)
45°C (Auto)	0.74	3.21	2.78	2.31	0.23
55°C (Auto)		2.97	2.85	1.85	
45°C	1.02	2.52	2.76	3.78	1.16
55°C		3.43	2.43	0.81	

Laccase activity of *Agaricus bisporus* (U3) in colonised compost (micro-moles/O_2/g dry matter), 21 days after inoculation. (Auto - composts autoclaved before inoculation).

Composting temperature	Control	+1 week	+2 weeks	+3 weeks	Lsd (5%)
45°C (Auto)	0.46	6.55	3.13	2.01	1.67
55°C (Auto)		5.87	3.42	0.91	
45°C	0.64	2.95	1.20	1.57	1.66
55°C		4.84	1.52	0.36	

Plate 3. Comparison of mycelial growth of *A.bisporus* (U3) in sterile and unsterile composts that had received prolonged composting times at 45°C and 55°C for 1 week (Expt. 8).

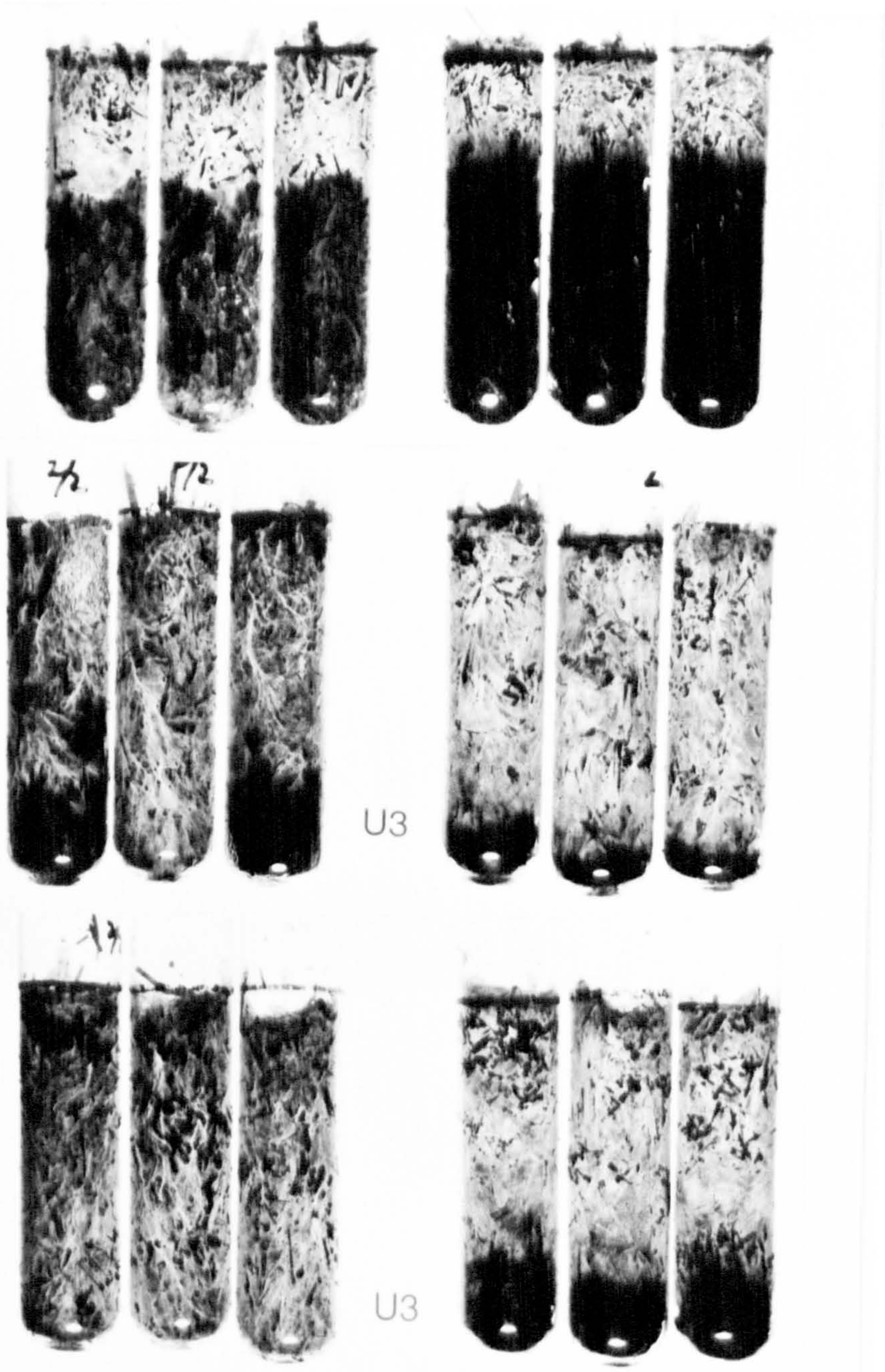


Figure 10. Comparisons of rate of mycelial colonisation in compost with laccase activity and ammonium ion concentration

(Experiment 8 - Prolonged composting at 45°C)

H.R.I. compost

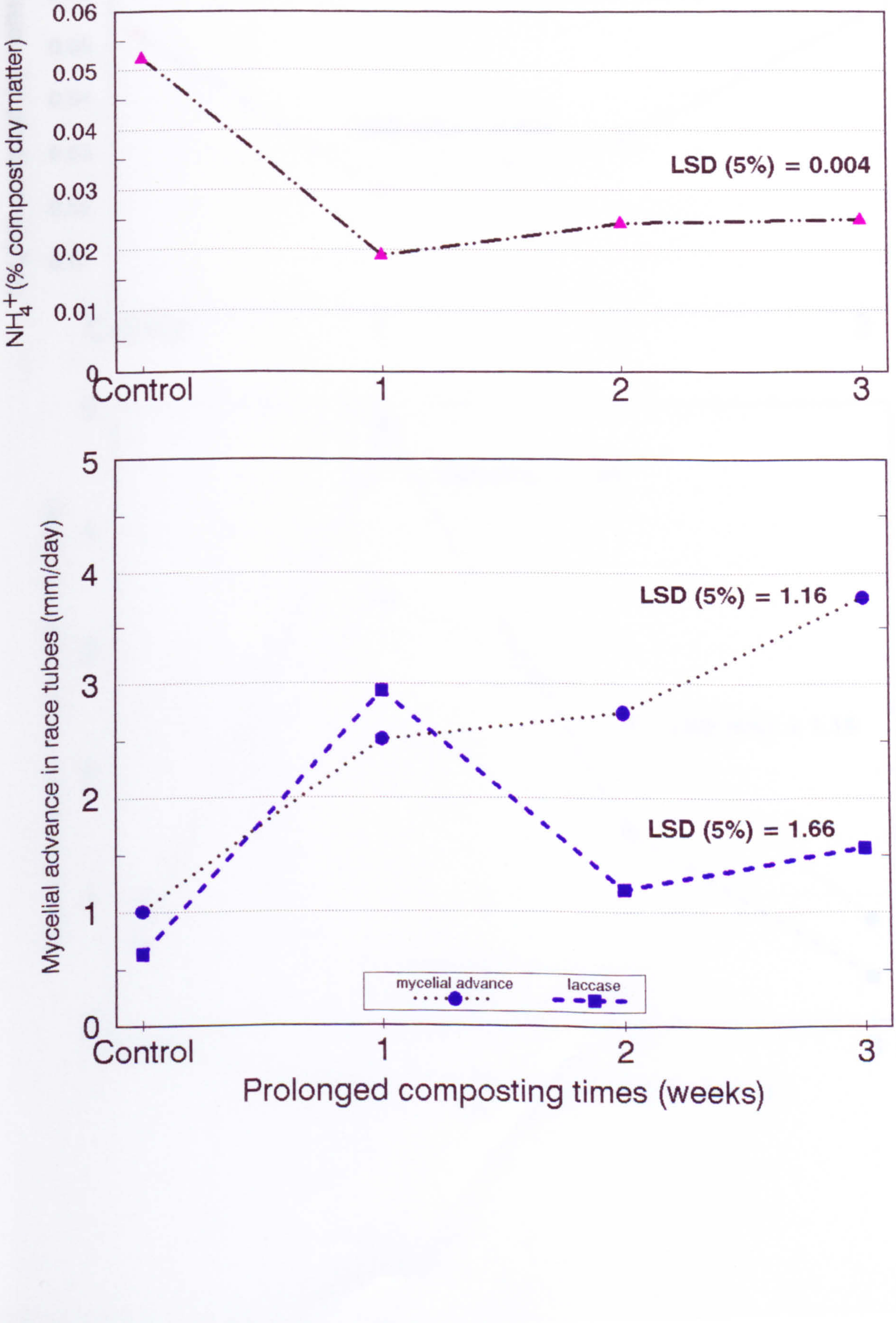


Figure 11. Comparisons of rate of mycelial colonisation in compost with laccase activity and ammonium ion concentration

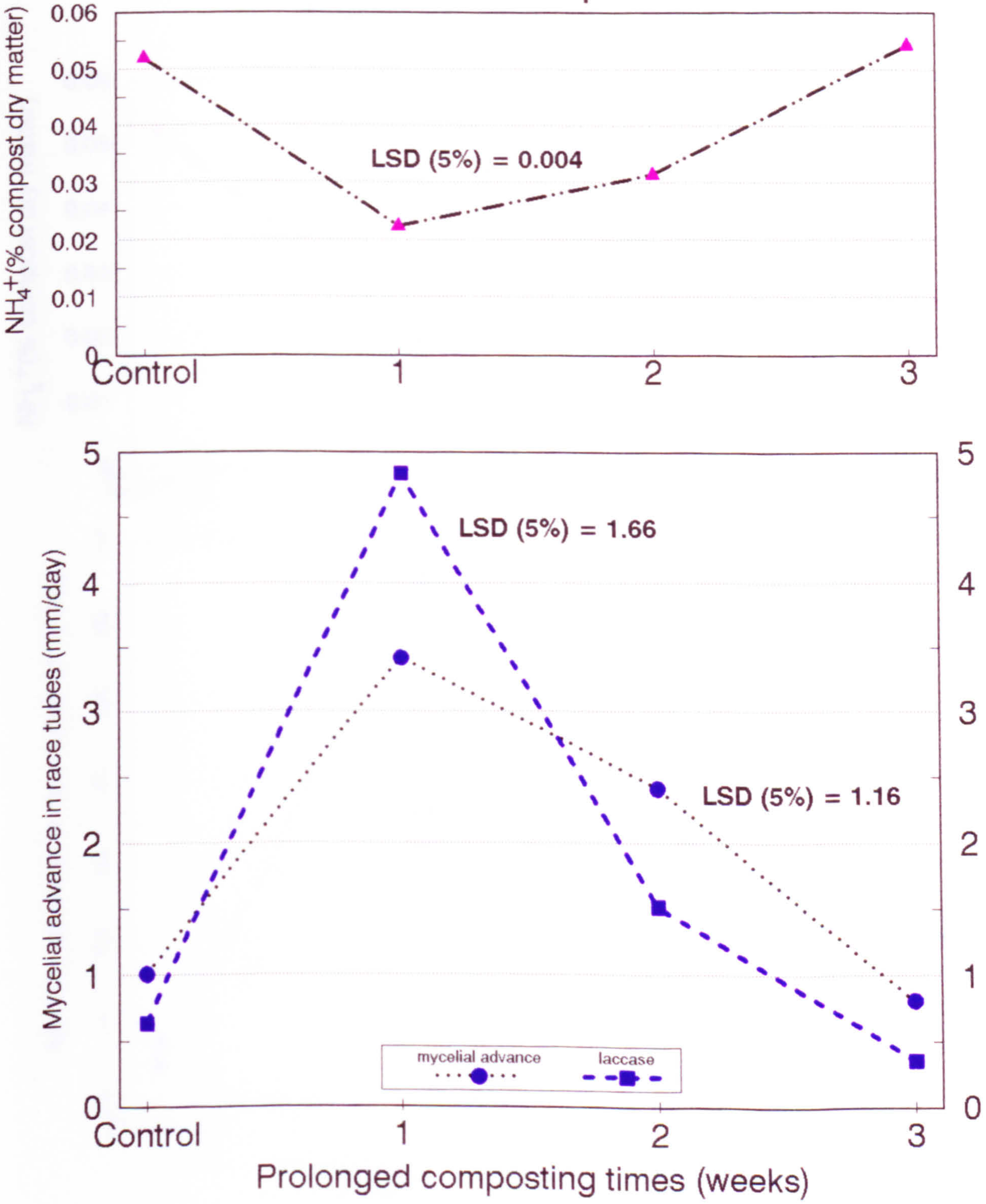


Figure 12. Comparisons of rate of mycelial colonisation in compost with laccase activity and ammonium ion concentration

(composts autoclaved before inoculation)

(Experiment 8 - Prolonged composting at 45°C)

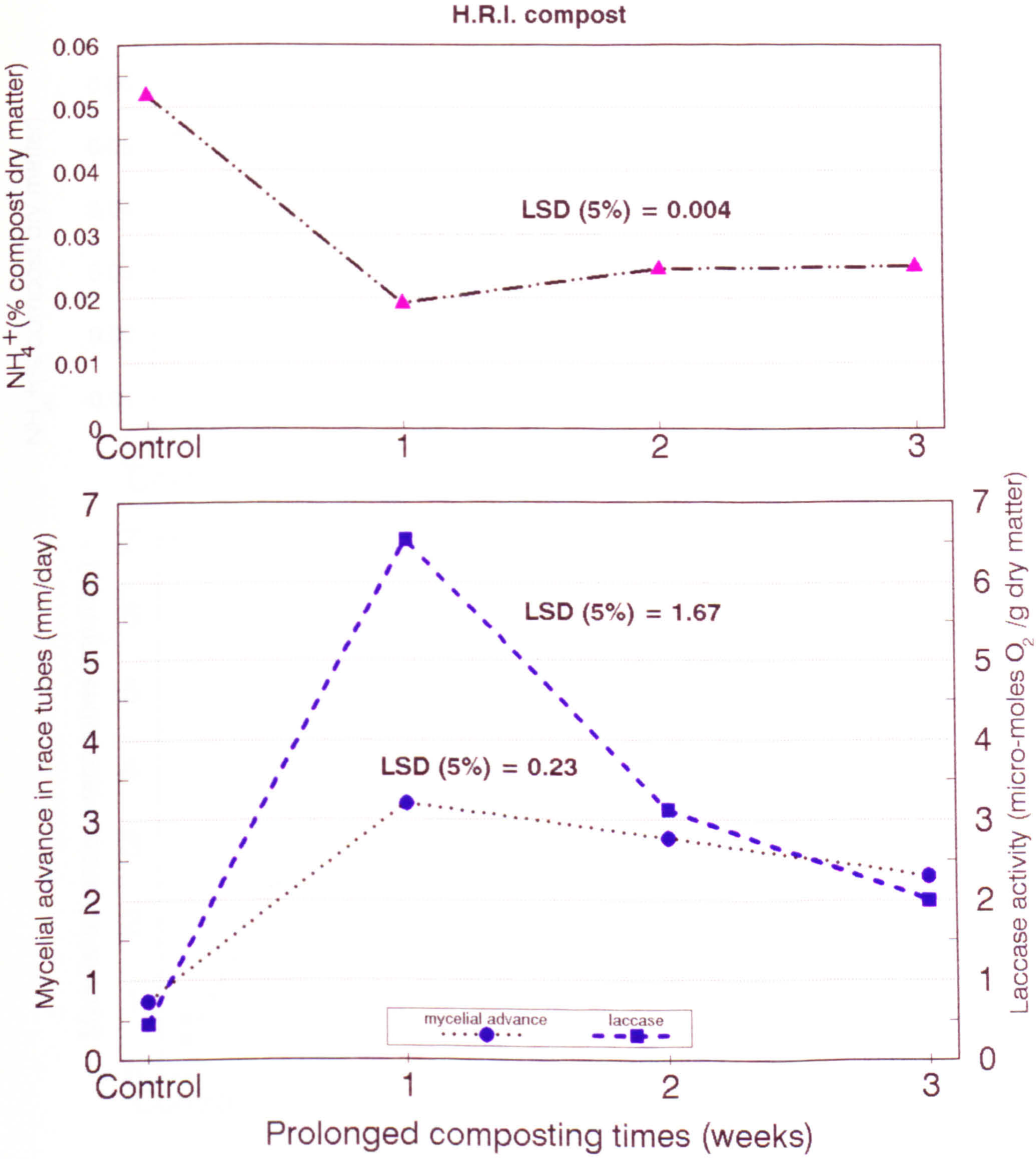
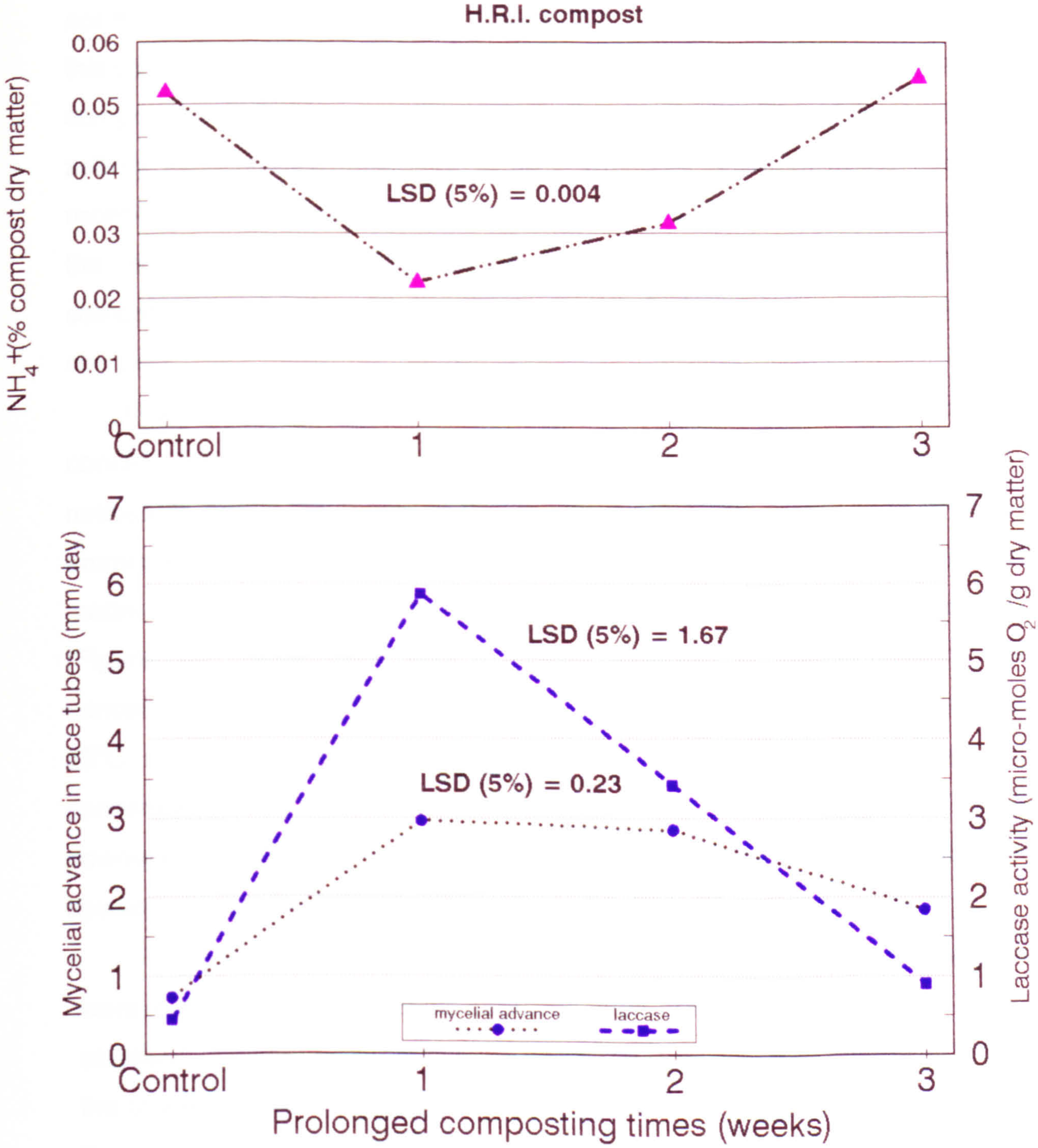


Figure 13. Comparisons of rate of mycelial colonisation in compost with laccase activity and ammonium ion concentration

(composts autoclaved before inoculation)

(Experiment 8 - Prolonged composting at 55°C)



Mycelial growth rate was also improved significantly in composts receiving an extended composting time of 55°C for 1 week but it steadily declined after 2 and 3 weeks composting (Figure 11). Laccase assays performed on these substrates generally showed an increase in activity in association with the mycelial growth rate but it was not always the case. The composts prolonged for 3 weeks at 45°C gave significantly the highest mycelial growth rate as measured by the mycelial advance in race tubes but not the highest laccase activity (Figure 10). The highest laccase activity for this compost was recorded from the sample receiving only 1 week's additional composting and this was also statistically significant. The highest laccase activity for composts prolonged at 55°C was also recorded in the compost receiving 1 week's additional composting (Figure 11). Laccase activity was the least in compost receiving an additional 3 week's composting and compared favourably with the poor growth of mushroom mycelium in this compost.

Freeze dried compost samples were also taken to assess the NH_4^+ ion concentration even though no large changes in pH were observed. It was noticeable that in the substrate that received prolonged composting at 45°C, ammonium concentration decreased from 0.053 to 0.019% of the compost dry matter after 1 week's composting but then remained essentially unchanged (Figure 10). While there was a similar decrease in the ammonium ion concentration in compost receiving an additional 1 week's composting at 55°C, in composts prolonged for 2 and 3 weeks at this temperature, the concentration rose to a level similar to the starting compost (Figure 11). The downward change in NH_4^+ ion concentration generally corresponded with the upward change in both mycelial colonisation rate and laccase activity.

The mycelial growth and laccase data presented in Figures 12 and 13 were obtained from sterilised compost treatments. Mycelial growth in the sterilised compost treatments were generally very similar to that obtained for the unsterile treatments with the exception of the control treatments, where the sterile control was significantly slower than the unsterile control (Plate 3). In the majority of cases, mushroom growth in sterile composts was more

uniform and visibly denser in comparison to the unsterile series. This is supported by the laccase activity data shown in Figures 12 and 13. Laccase activity was significantly higher in the compost treatments receiving 1 week's additional composting and where the ammonium ion concentration was at its lowest.

3.7 Experiment 9.

Experiment 9, resembled experiment 8, except that a well degraded commercial compost was used, prepared from stable manure supplemented with chicken manure (Blue Prince Mushrooms, Angmering, Sussex). This compost was extremely dark in colour when compared to the composts prepared using the HRI formulation reflecting a high degree of organic matter degradation (see also ash levels, Table 13).

As with previous experiments both mycelial growth through the composts, laccase activity and ammonium ion concentrations were monitored. In this experiment, it was quite clear that prolonging the composting time of this well degraded substrate at either 45°C or 55°C had little effect on mycelial growth rate and there were no significant differences in the levels of laccase activity recorded (Table 13) or ammonium ion concentrations (Appendix 9).

3.8 Experiment 10.

This experiment was conducted to address the question whether an improvement in mycelial colonisation rate and laccase activity by the mushroom could be used as indicators for an improved biological efficiency for the mushroom i.e. whether a faster mycelial colonisation of the substrate by the mushroom would result in an improvement in mushroom fruitbody production over a set period of cropping. The analytical changes are shown in Table 14.

In this experiment, to produce enough substrate to carry out productivity

Table 13. Analytical changes occurring within composts during prolonged composting periods at 45°C and 55°C and its effect on mushroom growth (Experiment 9).

Only one species *A.bisporus* (U3) was used in this experiment (Blue Prince Compost).

Treatment	Weight (g)	% H_20	Total dry matter (g)	% loss dry matter	% ash	Total N	pH
	Prolonged composting at 45°C						
Control	800.0	65.16	278.72	0	22.56	2.09	8.65
+1 week	786.4	71.16	226.79	18.63	24.34	2.48	8.70
+2 weeks	780.0	72.79	212.23	23.85	25.80	2.60	9.05
+3 weeks	765.5	75.47	187.77	32.63	30.68	2.65	8.30
Treatment	Prolonged composting at 55°C						
Control	800.0	65.16	278.72	0	22.56	2.09	8.65
+1 week	774.6	70.29	230.13	17.43	25.00	2.42	8.45
+2 weeks	769.8	74.69	194.83	30.09	27.36	2.89	8.20
+3 weeks	763.7	75.58	186.49	33.09	30.04	2.66	8.40

Mycelial advance (mm/day in 'Race tubes') of *A. bisporus* in composts receiving prolonged composting times at 45°C and 55°C.

Composting temperature	Control	+1 week	+2 weeks	+3 weeks	Lsd (5%)
45°C	6.80	6.60	5.58	6.73	0.23
55°C		6.96	6.23	6.71	

Laccase activity of *A.bisporus* (U3) in colonised compost (micro-moles O_2/g dry matter), 21 days after inoculation.

Composting temperature	Control	+1 week	+2 weeks	+3 weeks	Lsd (5%)
45°C	1.59	1.56	1.13	1.49	1.40
55°C		1.77	2.30	1.40	

Table 14. Analytical changes occurring within composts during prolonged composting periods at 45°C and its effect on mushroom growth (Experiment 10).

Treatment	Weight (g)	% H_20	Total dry matter (g)	% loss dry matter	% ash	Total N	pH
	Prolonged composting at 45°C						
Control	1000.0	75.88	241.20	0	16.16	2.38	8.25
+ 1 week	973.6	76.54	227.28	5.77	16.28	2.52	7.22
+ 2 weeks	961.62	77.76	213.86	11.33	17.60	2.76	7.17
+ 3 weeks	945.75	77.41	213.64	11.42	18.60	2.80	7.45

Mycelial advance (mm/day in 'race tubes') of *Agaricus bisporus*.

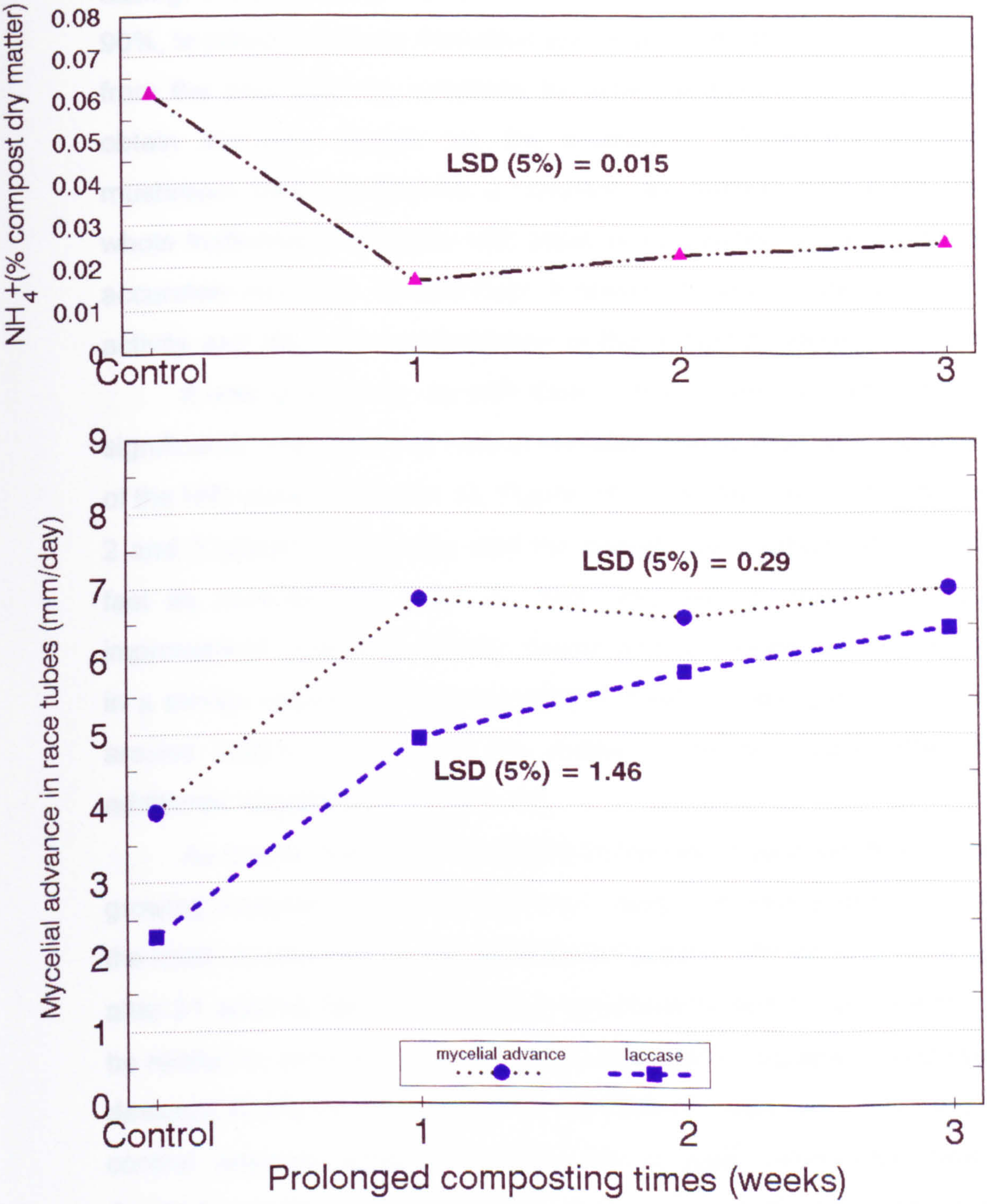
Composting temperature	Control	+ 1 week	+ 2 weeks	+ 3 weeks	Lsd (5%)
45°C	3.95	6.85	6.59	7.02	0.29

Laccase activity of *Agaricus bisporus* (U3) in colonised compost (μ moles O_2/g dry matter), 21 days after inoculation.

Composting temperature	Control	+ 1 week	+ 2 weeks	+ 3 weeks	Lsd (5%)
45°C	2.29	4.94	5.88	6.45	1.46

Figure 14. Comparisons of rate of mycelial colonisation in compost with laccase activity and ammonium ion concentration

(Experiment 10 - Prolonged composting at 45°C)



tests only one temperature, 45°C, was chosen for the prolonged composting period enabling 2 flasks each containing 1 Kg of fresh weight substrate to be used. After prolonged composting periods of 1, 2 & 3 weeks, plastic pots (10 x 10 x 12.5 cm deep), each containing 250 g of compost were inoculated with mushroom spawn (1% by weight), and the pots incubated at 25°C for 14 days before casing with a mixture of peat and chalk (3:1 ratio). After casing, the pots were maintained at 18°C (±0.5°C) at a relative humidity of 90%, to initiate fruitbody formation and mushroom fruitbodies were recorded from the pots until the substrate became exhausted (after 12 weeks). To obtain accurate figures for the production efficiencies achieved (i.e. mushroom fresh weight/100 g compost dry matter) from each treatment, whole fruitbodies (complete with stipe) were cleaned of casing material and accurately recorded. As with Expt. 8, speed of mycelial colonisation, laccase activity and NH_4^+ ion concentration of the composts were also determined.

It was quite clear, as with Expt. 8 that 1 week's composting at 45°C significantly improved the rate of mycelial colonisation and laccase activity of the HRI compost (Table 14; Figure 14). This improvement continued after 2 and 3 weeks composting and the overall colonisation rate was twice as fast as recorded for Expt. 8. This response is mirrored by a similar improvement in laccase activity. Ammonium ion concentration also behaved in a similar manner to that recorded in Expt. 8, falling from an initial level around 0.06% of compost dry matter to below 0.02% after 1 weeks additional composting (Figure 14).

As it is economically not viable to harvest mushroom from commercial growing systems (bags/shelves/trays) beyond 6 weeks of the first harvest, the main interest was to calculate the production efficiency of each substrate after 21 and 42 days of harvesting mushrooms and to see whether it could be related to an improvement in the colonisation rate and/or laccase activity detected during the spawn run. As the highest yields were recorded from the control compost which had given the slowest colonisation rate, it was decided to continue harvesting mushrooms for a further 6 weeks from all the treatments, to a time when the substrate had lost its selectivity and was

possibly nutritionally exhausted. Mushroom yields together with the calculated production efficiencies are highlighted in Figure 15 (see also Appendix 11).

3.9 Mycelial growth on 2% malt agar supplemented with increasing concentrations of ammonium salts.

Observations made regarding speed of mycelial colonisation in a range of composts prepared by prolonging the composting period identified a possible relationship with the ammonium ion concentration at the time of spawning (see Expts. 8, 9 & 10). To confirm that this observation was a real one, mushroom mycelium was grown on 2% malt agar (buffered) supplemented with increasing amounts of an ammonium salt. Initially ammonium sulphate was used at increasing levels (0, 250, 500, 1,000 and 2,000 ppm NH_4^+) and 8 replicate plates were prepared for each treatment. This experiment was performed twice. A further experiment was also carried out using ammonium tartrate as the source of NH_4^+ as this salt has less tendency to acidify the medium. The results of these three experiments are graphically illustrated in Figure 16. While there were wide differences in the radial growth rates between respective treatments for each experiment, which can possibly be explained by the age of the culture inoculum used, it was quite clear that the radial growth of mushroom mycelium was significantly affected by an increasing presence of the NH_4^+ ion (Plate 4). Agar surface pH measurements were done on all treatments after 26 days and were found to be all within the range 6.1-6.2.

3.10 The effect of an increasing NH_4^+ ion concentration on fungal biomass accumulation and laccase activity in liquid culture.

The previous experiment on agar medium showed a clear effect of increasing NH_4^+ on colony radial growth. Although it maybe that a slower

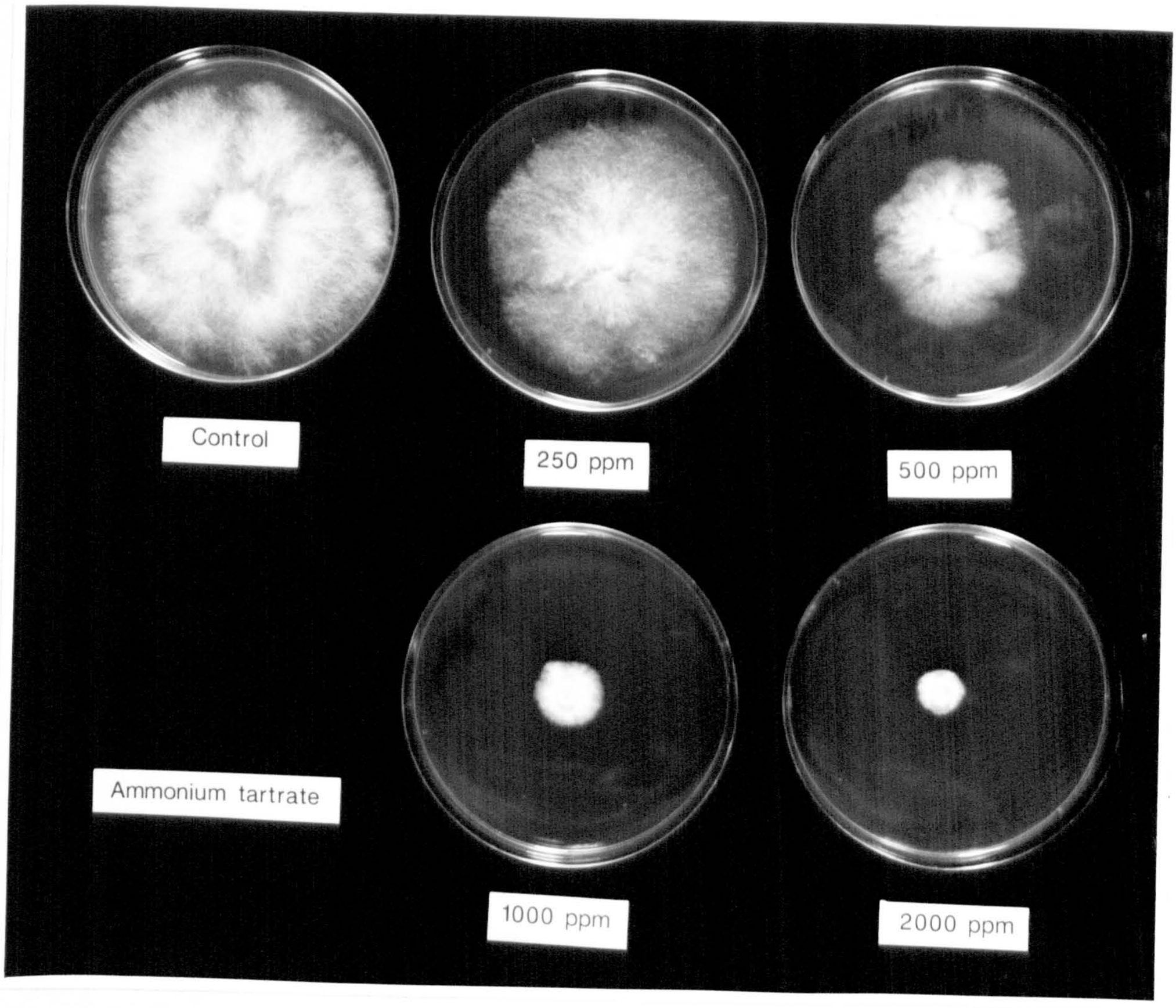


Plate 4. Effect of increasing ammonium ion concentrations (ammonium tartrate) on radial growth of *Agaricus bisporus* (U3) on 2% malt agar (buffered)

Figure 15. Fresh weight mushrooms harvested per 100g compost matter (Production Efficiency) from 4 compost types

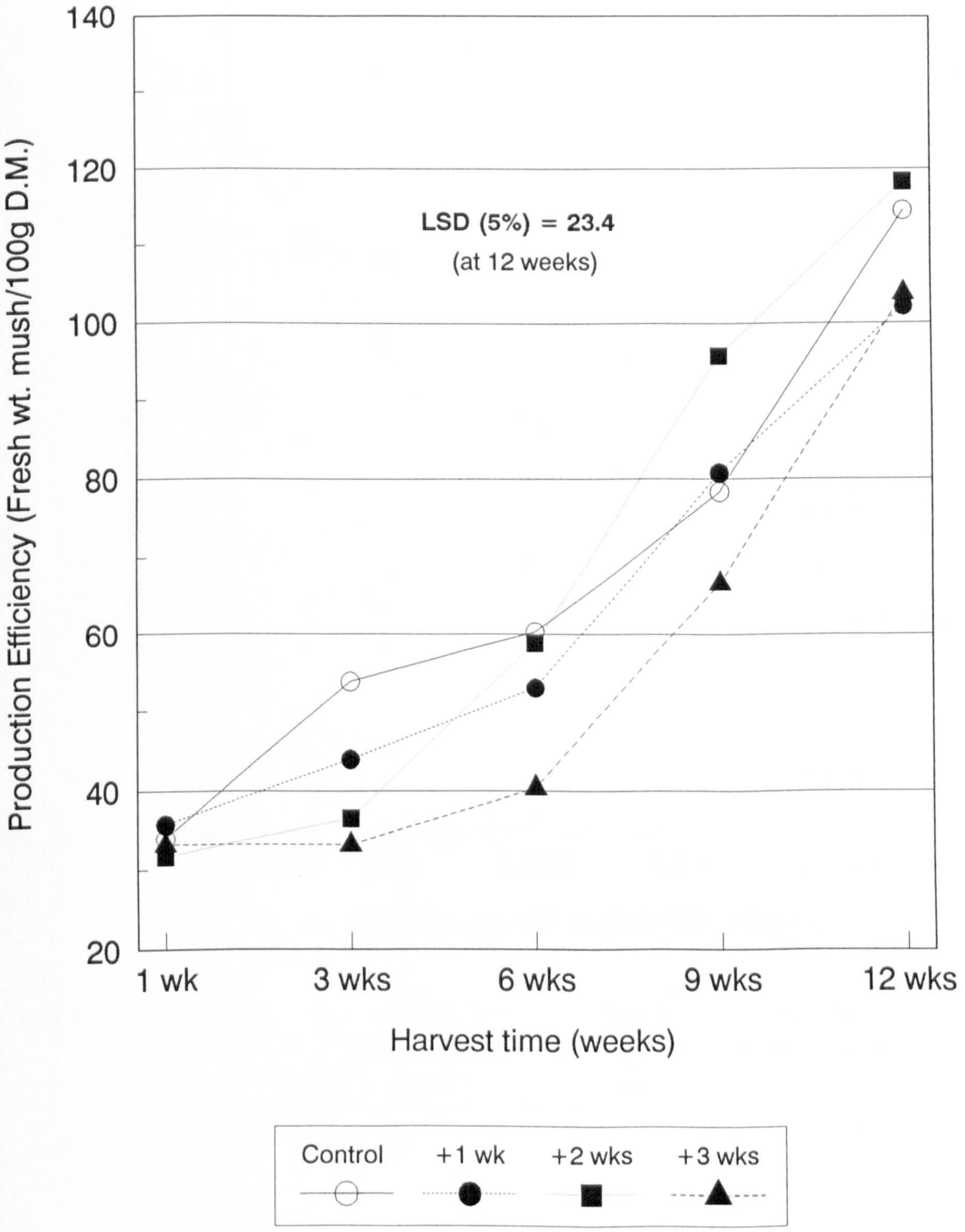
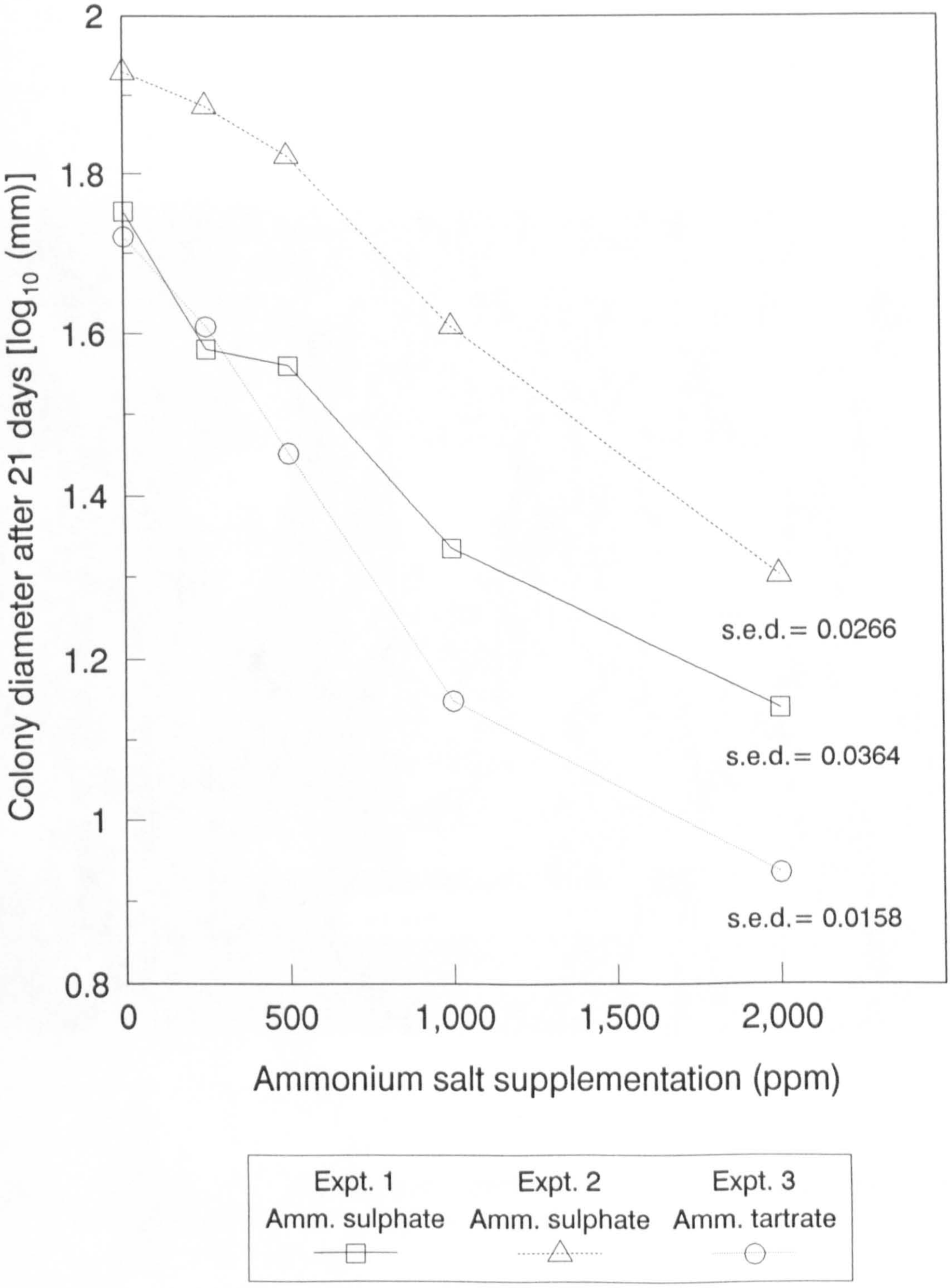


Figure 16. Mycelial Growth on 2% Malt Agar supplemented with ammonium salts



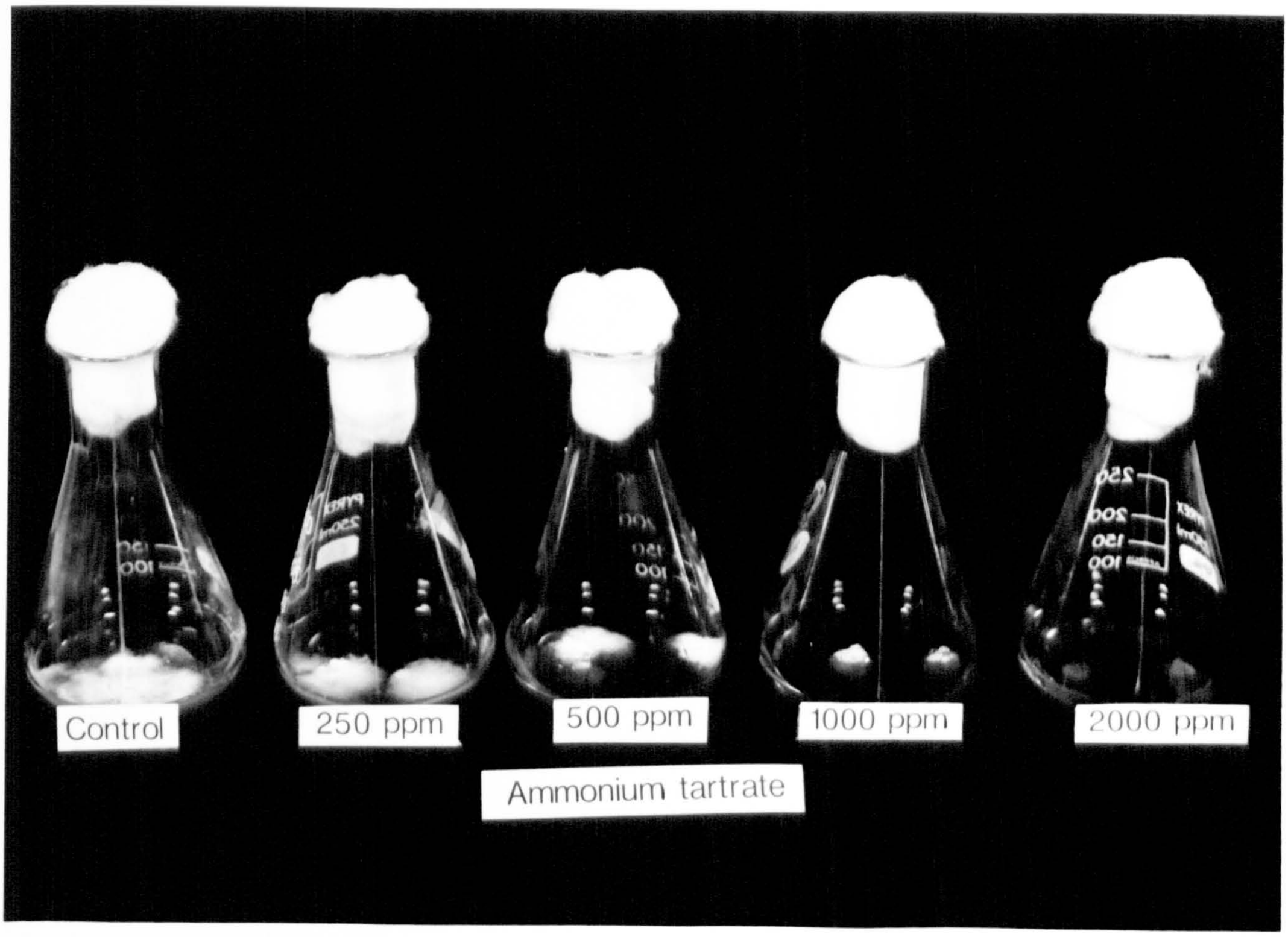
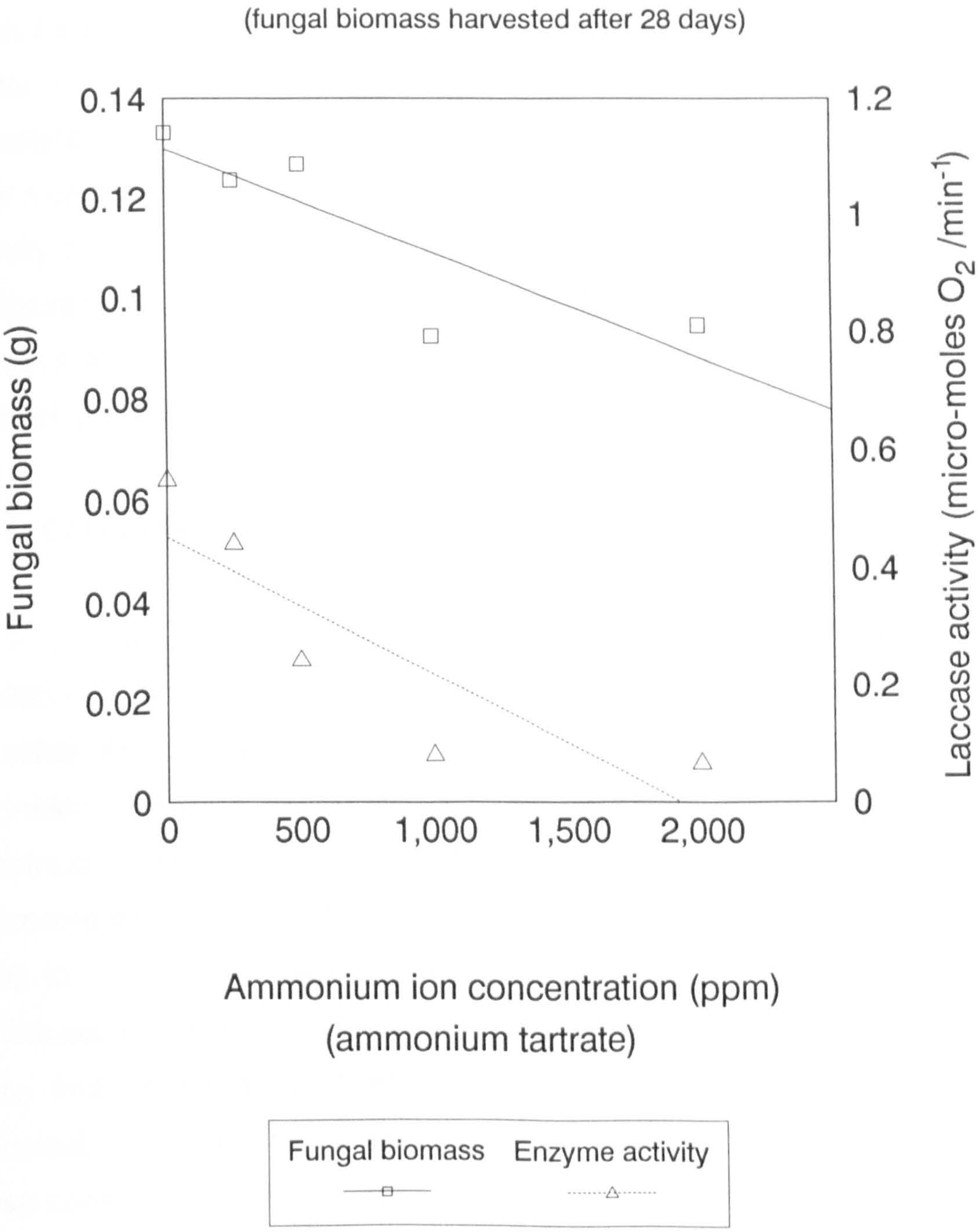


Plate 5. **Effect of increasing ammonium ion concentrations (ammonium tartrate) on fungal biomass (*A. bisporus*; U3) in liquid culture**

Figure 17. The effect of increasing NH_4^+ ion on fungal biomass and laccase activity in liquid culture



growing colony produced less fungal biomass in a given time than a faster growing one, it is not always so. To confirm that an increasing ammonium ion concentration reduced mycelial biomass accumulation, an experiment was performed in liquid culture (2% buffered Malt Extract medium) using 5 treatments (0, 250, 500, 1,000 and 2,000 ppm NH_4^+). Ammonium tartrate was used as the NH_4^+ source and 5 replicate flasks each containing 40 ml of media were set up and two mycelial plugs (5mm diam) were added to each flask. The growth of mycelium in the control medium was visibly much faster than all other treatments (Plate 5) with surface mycelium being apparent after 16 days. The fungal biomass that accumulated after 28 days was significantly reduced by an increasing NH_4^+ concentration and laccase activity measured in the culture was also significantly reduced (Figure 17). Measurements of pH were done on all supernatants of the 5 treatments after 28 days and were found to be 6.2 in the control flasks falling to 5.85 in the highest ammonium tartrate treatment.

3.11 Concluding Comments

It was quite clear from the first experiments that subjecting a traditionally prepared mushroom substrate to extended composting periods at either 45°C or 55°C not only, as would be expected, produced more degraded substrates but the rate at which mushroom mycelium colonised its substrate, was significantly affected for a number of species. While improvements in colonisation rate were observed for composts prolonged at 45°C for 1, 2 and 3 weeks, in composts prolonged at 55°C a decline in colonisation rate was common after 2 weeks composting. Laccase assays were first performed in Expt. 8 to determine the relationship between colonisation rate and fungal biomass. While there would appear to be a very close correlation for the majority of composts tested, in some cases laccase activity, and thereby fungal biomass, was shown to decline while the mycelial colonisation rate increased (Figure 10). It might be concluded that the greater the nutrient status of a substrate, the greater the rate of mycelial extension

away from it would be. However, in some cases e.g. wood-decaying *Basidiomycotina,* outgrowths of mycelium into non-sterile soil from a wood block inoculum occurs by means of well differentiated mycelial cords (Thompson 1982; Thomspon & Rayner, 1982). In contrast, outgrowths from the inoculum source into sterile soil was shown to be by means of a more diffuse mycelium that advances at a lower rate than do cords in non-sterile soil. The dry matter loss from the wood block inoculum was also found to be greatest in the sterile soil which suggested that in generally high nutrient conditions (as in sterile soil free of microflora; nutrients freely available) there is a tendency for the mycelium to dwell.

In ecological terms therefore, a fast-effuse growth of *A. bisporus* mycelium may be explained by mycelial exploration and occupation of a nutritionally poor substrate, whilst a slower denser growth can be explained by a consolidation of captured territory and exploitation of the energy resources therein. It can be concluded that mycelial growth rates alone cannot be used as an indicator of mycelial establishment and wherever possible laccase assays should be carried out to give a more positive assessment of fungal biomass.

One observation which required further investigation was a possible correlation between the NH_4^+ concentration at the time of spawning and laccase activity shown after 3 weeks mycelial colonisation of the substrate. Laccase activity was at its highest in composts receiving one weeks prolonged composting, irrespective of temperature regime, and in composts with the lowest NH_4^+ concentration at spawning. It was also noticeable (Figure 11) that when the NH_4^+ concentration increased by prolonging composting at 55°C both laccase activity and mycelial colonisation rate declined. While a similar pattern of results were obtained in composts that were sterilised before inoculation, a higher laccase activity was recorded after 1 week's prolonged composting, which suggested that a greater fungal biomass had accumulated in comparison to the unsterilised series. It can be reasoned that

such an improvement was achieved by removal of competing micro-organisms allowing a more efficient degradation of the substrate.

Experiment 9 was performed to see whether the observations made with a H.R.I. compost would be true for a commercial compost obtained from a local mushroom farm (Blue Prince, Poling, West Sussex). While the extended composting regime at 45°C and 55°C for 1, 2 and 3 weeks was applied to this compost, no significant increases or decreases were observed in colonisation rate or laccase activity. As the NH_4^+ concentration also remained at a constant low level i.e. 0.03% of the compost dry matter, the apparent correlation between a low NH_4^+ concentration and high colonisation rate and laccase activity is possibly a true one.

Experiment 10 was virtually a repeat of Experiment 8a (i.e. extended composting periods at 45°C) but was conducted specifically to test whether colonisation rate differences induced in mushroom composts could be related to the ultimate yield of mushrooms obtained from that substrate. Once again mycelial colonisation rate was significantly improved by prolonging the composting time for 1 week. Further extension of the composting period had little effect on the colonisation rate although laccase activity within the substrate increased steadily with time. An association between the significant reduction in the NH_4^+ concentration after 1 week of extended composting and an increased colonisation rate and laccase activity once again appeared genuine. Perhaps the most surprising result obtained from Experiment 10 was that there were no significant differences in mushroom yield between compost types irrespective of the noted wide differences of colonisation rate shown by 'race tube' assessment.

The effect of increasing levels of the ammonium ion (250 - 2,000 ppm) on the rate at which mushroom mycelium colonised its substrate was determined on both solid (See 3.9) and liquid (See. 3.10) media. Both ammonium sulphate and ammonium tartrate were used as a source of

ammonium ion and in all three experiments where these salts were included in a buffered agar medium it was quite clear that the mushroom mycelium was sensitive to ammonium levels above 500 ppm. This is comparable with the data obtained in Expts. 8, 9 & 10 where mycelial colonisation rates were improved when the levels of ammonium ion were below 0.05% of the compost dry matter i.e. 500 ppm of compost dry matter. The liquid culture study also confirmed earlier findings in that fungal biomass accumulation and laccase activity were reduced significantly in response to an increasing ammonium ion presence.

CHAPTER 4 ASSESSMENT OF HUMIC MATERIALS THAT ACCUMULATE DURING THE COMPOSTING PROCESS AND THEIR ROLE IN MUSHROOM NUTRITION

4.1 Development of a reliable extraction procedure to assess the alkali soluble/acid insoluble fraction (humic acid) of composted substrates.

Eddy (1976) employed a Soxhlet extraction procedure, using hot dilute sodium hydroxide for several hours, to extract the so called 'dark layer' that closely adheres to the cereal straw surfaces in traditionally prepared mushroom composts. Wain (1981) questioned this procedure as the the total amount of extracted material was found to be in around 50% of the ash free compost at spawning (30% soluble carbohydrate, 20% humic fraction). This extraction method was considered severe as solubilisation of the plant polymers was very likely to occur at high temperatures making subsequent estimation and analysis of the extracted fractions misleading. Wain (1981) investigating less severe procedures, found that sonication of freeze dried compost samples in cold water or dilute alkali, was a less severe and more reliable way of extracting the dark humic fraction. Dilute alkali e.g. 0.1 to 0.5N NaOH has been a popular extractant of soil organic matter for decades (Stevenson, 1982). Wain (1981) demonstrated that extraction of the alkali-soluble/acid-insoluble fraction could be carried out quite efficiently using 0.5M NaOH and a short sonication time of 10-15 minutes, without altering the chemical constitution of the material. He identified two distinct fractions (a) the alkali-soluble/acid-insoluble fraction or humic component which represented about 18% of compost dry matter and (b) the debris fraction insoluble in dilute alkali which represented about 12% of compost dry matter. To confirm these findings a number of experiments were performed, using sonication and dilute alkali, to assess the efficiency of release of the humic material from the cereal straw component and also the solubilisation of plant polymers with time of treatment.

4.2 Sonication time experiments S1 - S4.

(Experiments S1 & S2)

In the first experiment sonication times of 4, 6, 8, 10, 12 and 14 minutes were chosen and the procedure as outlined in 2.9.1 (see Figure 3) was performed on a H.R.I. compost formulation that had employed recently harvested straw. The amounts of humic, debris 1 and debris 2 fractions remaining after sonication with 0.5 M NaOH were calculated by averaging the mean of two replicate starting samples. The residual fractions obtained after extraction are graphically represented in Fig. 18. There were no significant weight differences between the fractions obtained after 4, 6, 8, 10 and 12 minutes sonication and it was only after 14 minutes that a distinct decrease in the debris 1 fraction and increases in both the humic and debris 2 fractions were noted. This experiment was repeated using the same compost with sonication times of 8, 10, 12, 14 and 16 and a similar pattern of results was obtained. The debris I fraction was estimated to be between 70-80% of the compost dry matter whereas debris 2 and humic fractions were 8-11% and 3-7% respectively (these calculations were determined from the most consistent part of the graph.)

(Experiments S3 & S4)

In both the previous experiments, the shortest sonication treatments were performed first and to test whether the results were genuine differences caused by extraction time and not due to fluctuations in frequency output of the sonicator, two further experiments were conducted. On this occasion a darker, well degraded compost was used. Experiments S3 & S4 were conducted using identical sonication time treatments of 2, 4, 6, 8, 10, 12, 14, & 16 mins. The only difference between experiments was that in Expt.4, the sonication time treatments were performed in reverse order i.e. the longest

Figure 18. The effect of increasing sonication time on the estimation of humic, debris 1 & debris 2 fractions of freeze dried H.R.I. compost samples (Expts. S1 & S2).

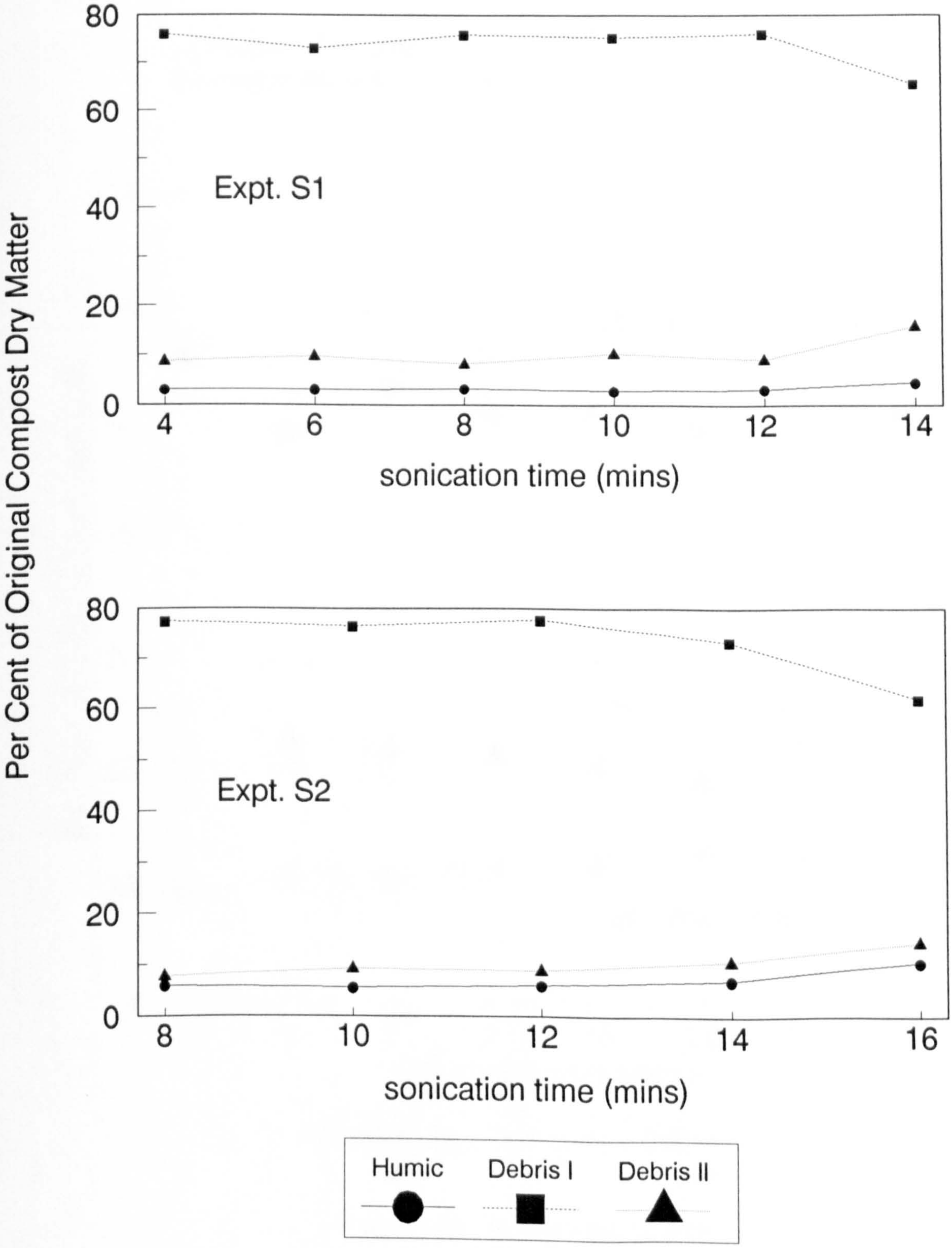


Figure 19. The effect of increasing sonication time on the estimation of humic, debris 1 and debris 2 fractions of H.R.I. freeze dried compost samples (Expts. S3 & S4)

(a) shortest extraction time first - S3
(b) longest extraction time first - S4

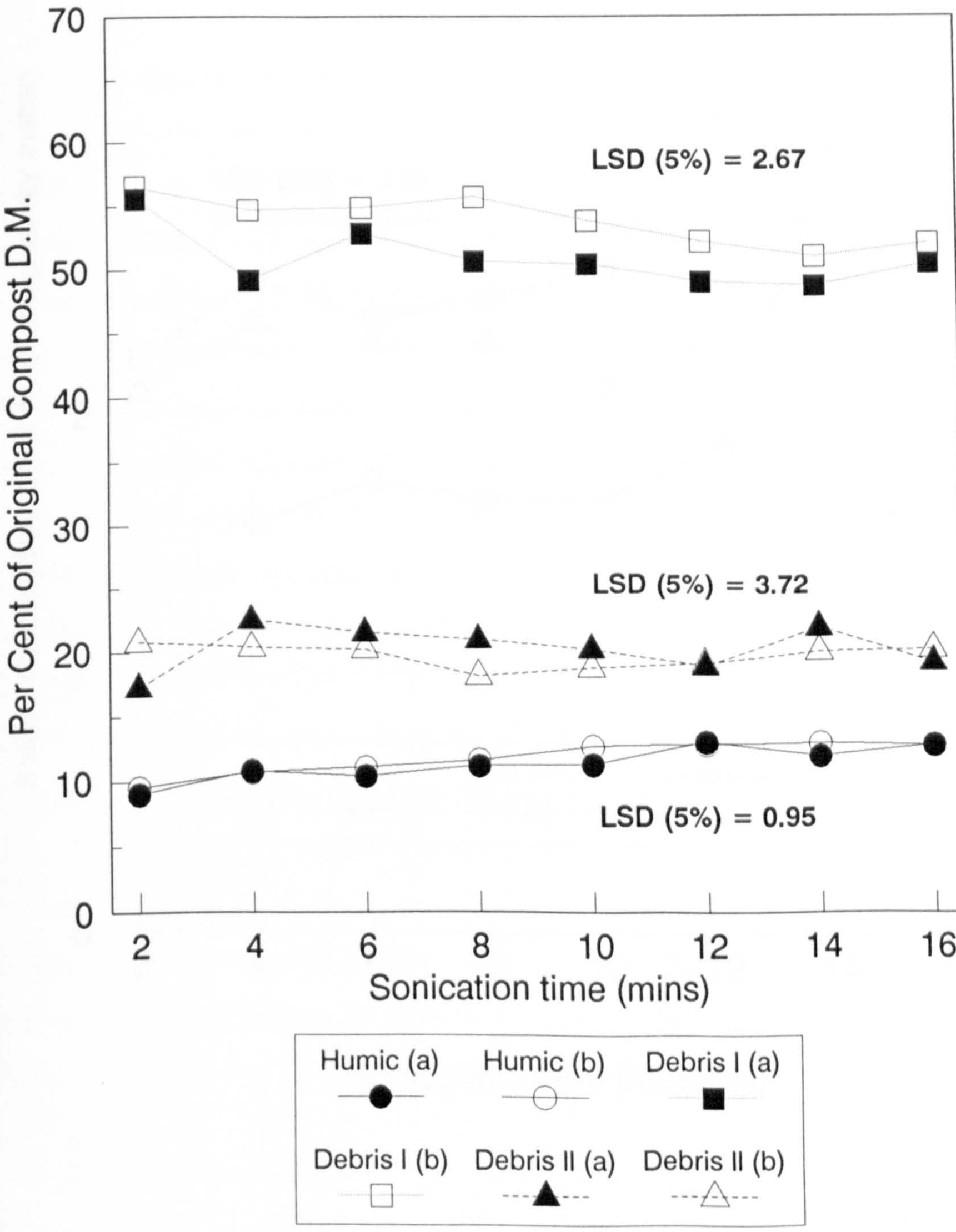
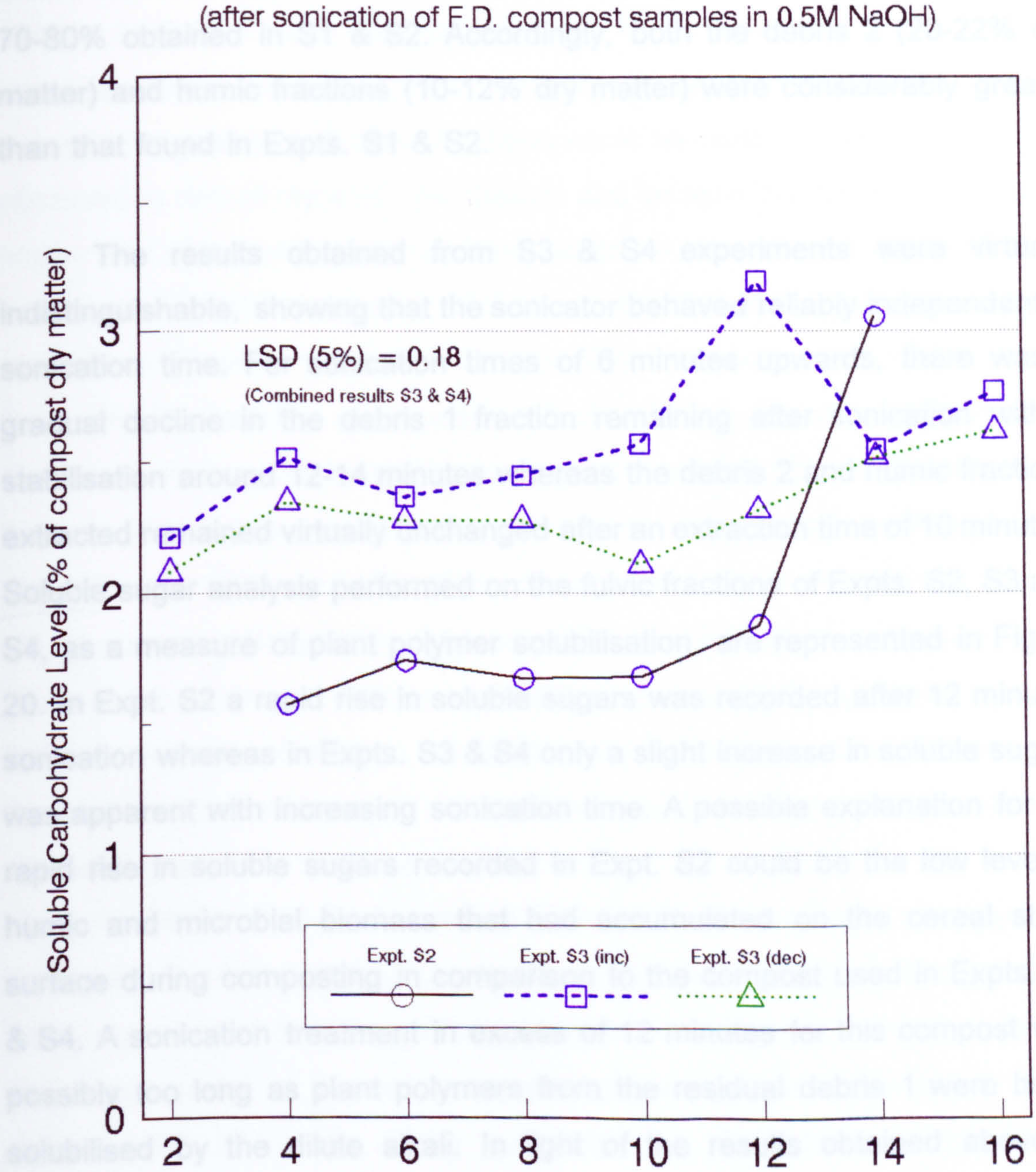


Figure 20. Analysis of Soluble Carbohydrates in Fulvic Fraction



sonication time first. The results of both experiments are graphically presented in Figure 19. The results confirmed that this compost was of a more degraded nature than the compost used in the previous two experiments as the debris 1 fraction (i.e. the straw component free of the humic material) represented about 55% of compost dry matter compared to 70-80% obtained in S1 & S2. Accordingly, both the debris 2 (20-22% dry matter) and humic fractions (10-12% dry matter) were considerably greater than that found in Expts. S1 & S2.

The results obtained from S3 & S4 experiments were virtually indistinguishable, showing that the sonicator behaved reliably independent of sonication time. For sonication times of 6 minutes upwards, there was a gradual decline in the debris 1 fraction remaining after sonication with a stabilisation around 12-14 minutes whereas the debris 2 and humic fractions extracted remained virtually unchanged after an extraction time of 10 minutes.

Soluble sugar analysis performed on the fulvic fractions of Expts. S2, S3 and S4, as a measure of plant polymer solubilisation, are represented in Figure 20. In Expt. S2 a rapid rise in soluble sugars was recorded after 12 minutes sonication whereas in Expts. S3 & S4 only a slight increase in soluble sugars was apparent with increasing sonication time. A possible explanation for the rapid rise in soluble sugars recorded in Expt. S2 could be the low level of humic and microbial biomass that had accumulated on the cereal straw surface during composting in comparison to the compost used in Expts. S3 & S4. A sonication treatment in excess of 12 minutes for this compost was possibly too long as plant polymers from the residual debris 1 were being solubilised by the dilute alkali. In light of the results obtained above, a maximum sonication time of 10 minutes was chosen to isolate the humic and debris 2 fractions for all further experiments.

4.3 Changes in the proportions of humic, fulvic, debris 1 and debris 2 fractions during prolonged composting of two distinct compost types (Expts. 8 & 9).

In Expt. 8 (H.R.I. Compost), notable changes in mycelial growth rate and laccase activity were reported in composts that were prolonged at either 45°C or 55°C for 1 week. Estimations of the humic, fulvic and debris fractions were assessed on freeze dried compost samples to determine whether proportional changes of these fractions could be related to distinct changes observed in rate of mycelial colonisation and laccase activity. As the organic matter was consumed by the resident microflora, the debris 1 (straw fraction) was shown to fall from around 60% to around 40% of the total compost dry matter over the three week period (Figure 21), irrespective of the temperature treatment. Degradation of the cellulose/hemicellulosic constituent of the straw fraction is also shown by the increasing ash contents (Table 12). While the humic and fulvic proportions remained reasonably unchanged at approximately 10% and 15% of the compost dry matter respectively (Figure 21), the most noticeable change occurred with the debris 2 fraction which increased from around 15% to over 30% of the compost dry matter over the three week period. However, no direct relationship between this fraction and the improved mycelial growth rate observed in composts receiving additional composting periods could be made.

Analysis of both the humic and fulvic fractions for protein, carbohydrate and phenolic content are shown in Figures 22 & 24. It was quite clear from the analyses of all the compost types produced in Expt. 8 that protein was a major constituent of the humic complex (Figure 22), in some cases being close to 50% of the dry matter in this fraction. The soluble carbohydrate component of the humic complex was found to be between 15-20% while the phenolic component was about 10% of the compost dry matter. While there would appear to be differences in the proportions of these three fractions in the range of composts produced, especially the protein constituent, no correlation could be made between the component fractions and rate of

Figure 21. Effect of prolonged composting times on the proportions of debris 1, debris 2, humic and fulvic fractions of composts

(Experiment 8 - composts prolonged at 45℃ and 55℃)

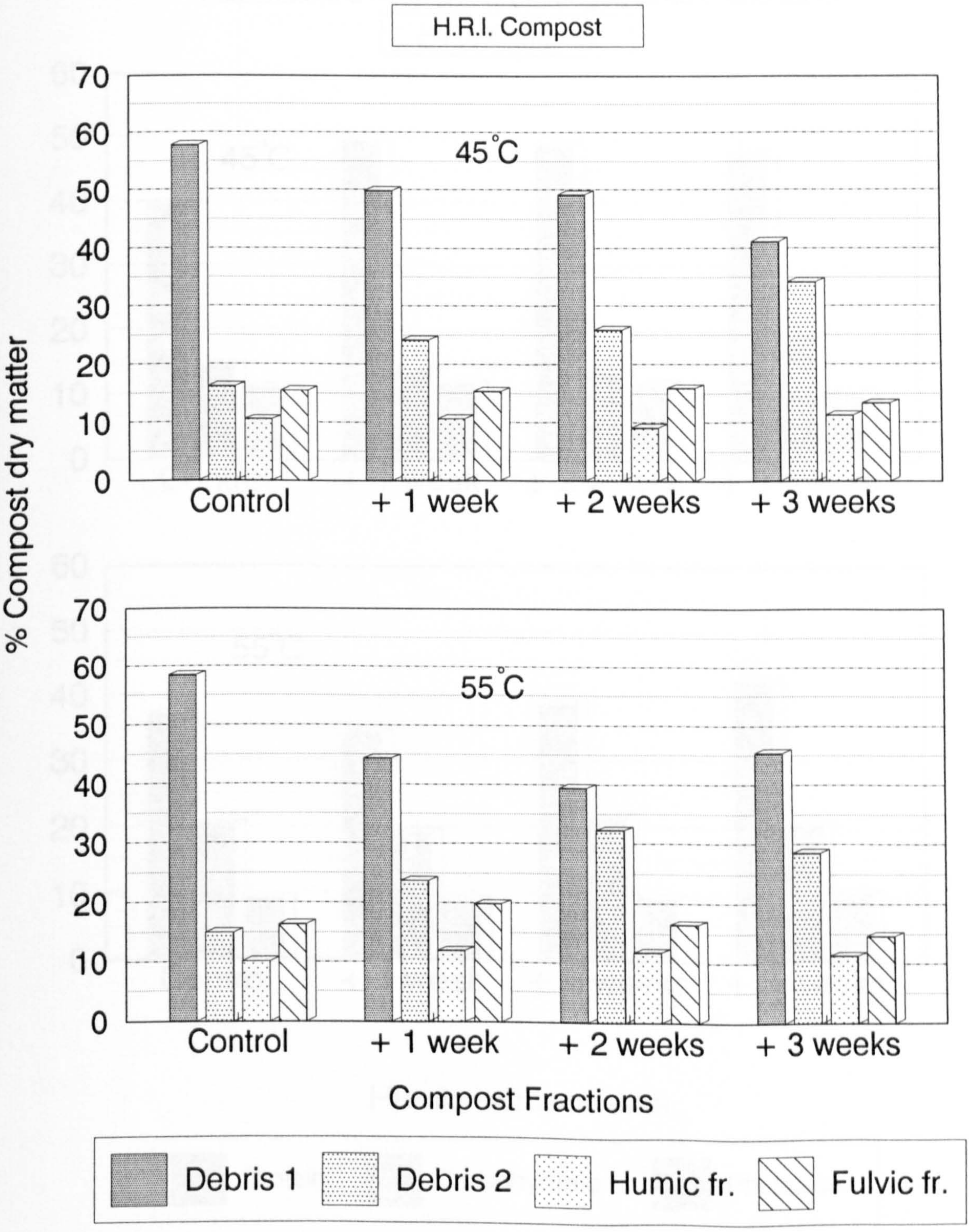


Figure 22. Protein/Carbohydrate/Phenolic content of Ash-Free Humic Material

(Experiment 8 - composts prolonged at 45°C and 55°C)

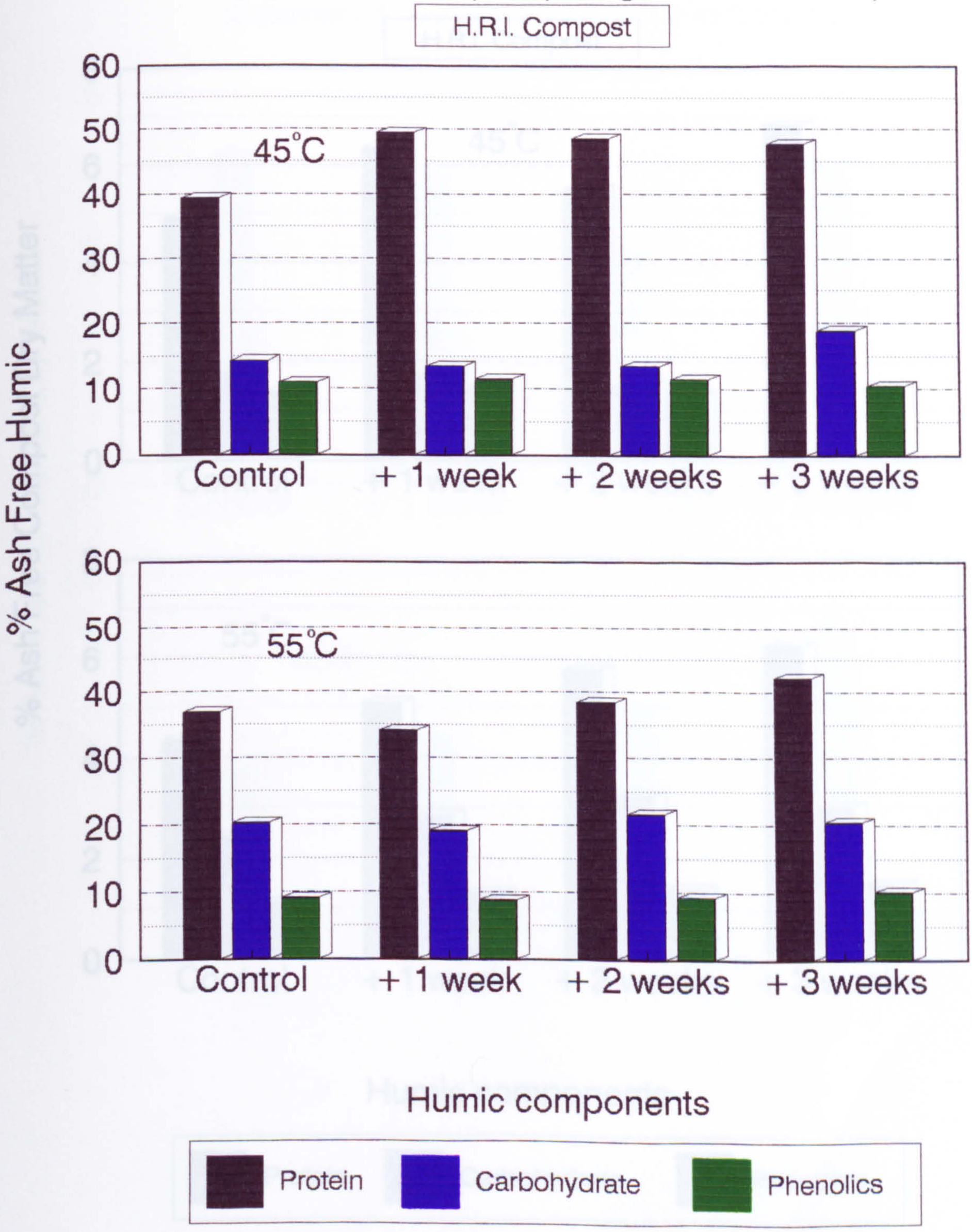


Figure 23. Protein/Carbohydrate/Phenolic content of Humic Fraction
(as a %age of ash-free compost dry matter)

(Experiment 8 - composts prolonged at 45℃ and 55℃)

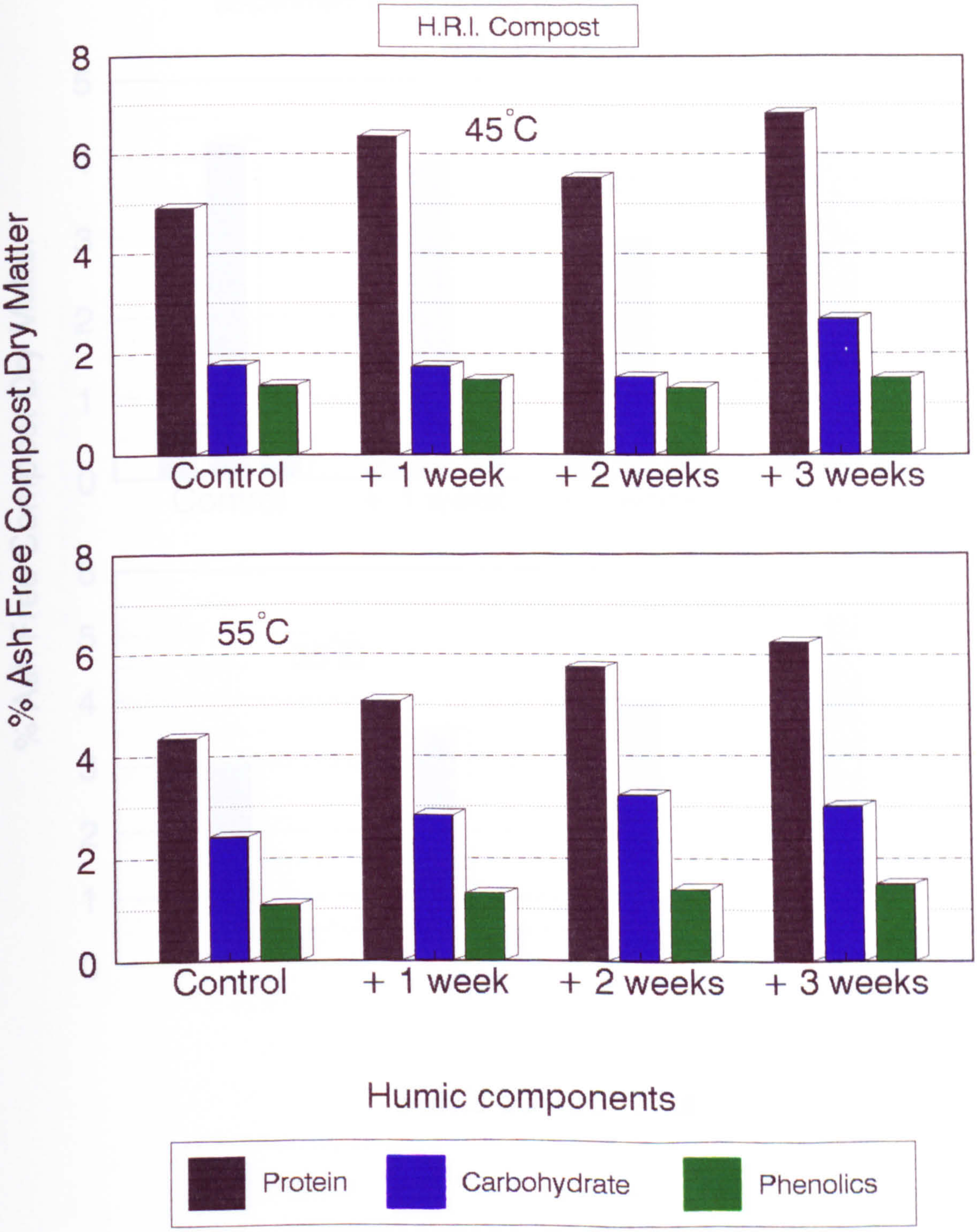


Figure 24. Protein/Carbohydrate/Phenolic content of Fulvic Fraction (as a %age of ash-free compost dry matter)

(Experiment 8 - composts prolonged at 45°C and 55°C)

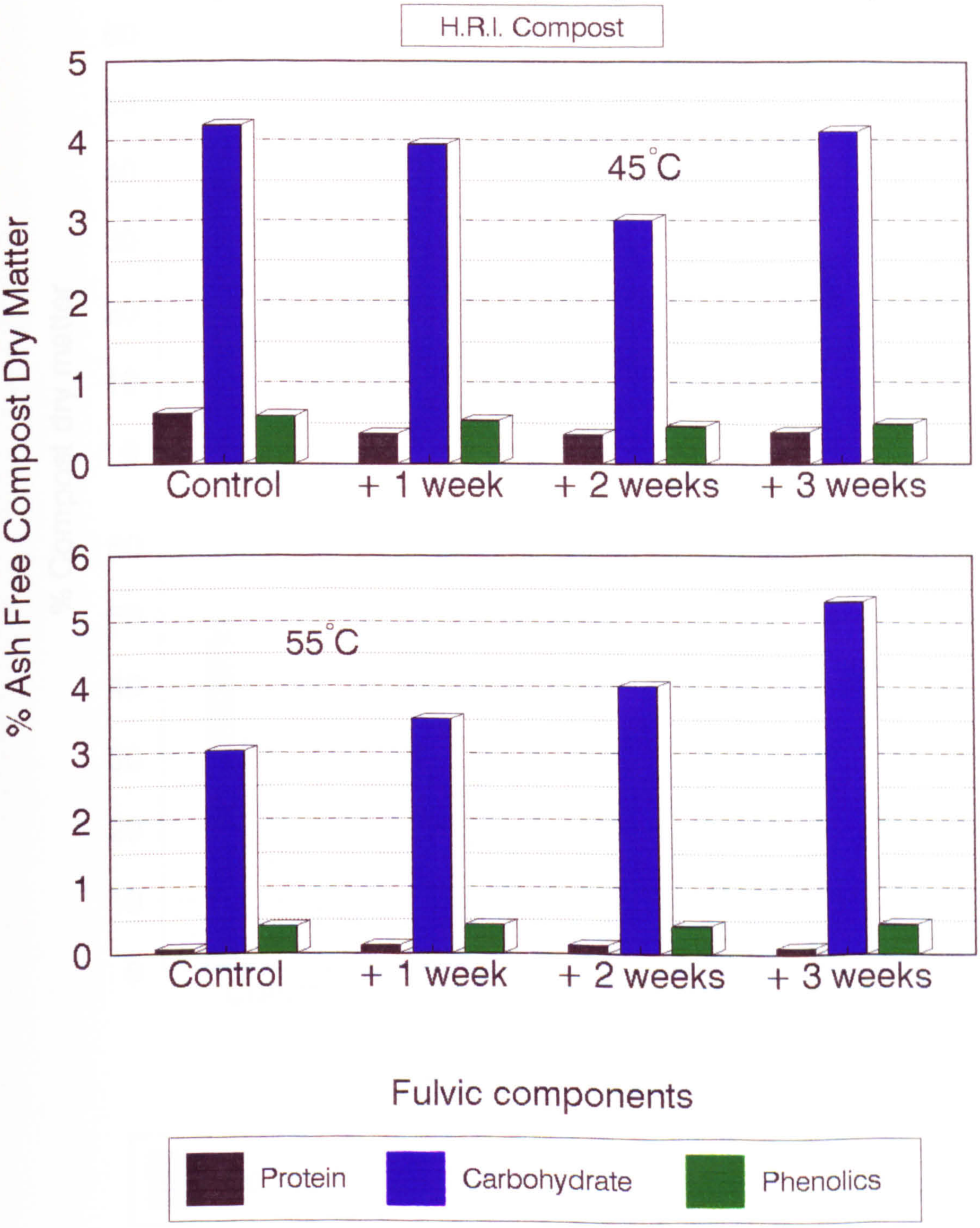


Figure 25. Effect of prolonged composting times on the proportions of debris 1, debris 2, humic and fulvic fractions of composts

(Experiment 9 - compost prolonged at 45°C and 55°C)

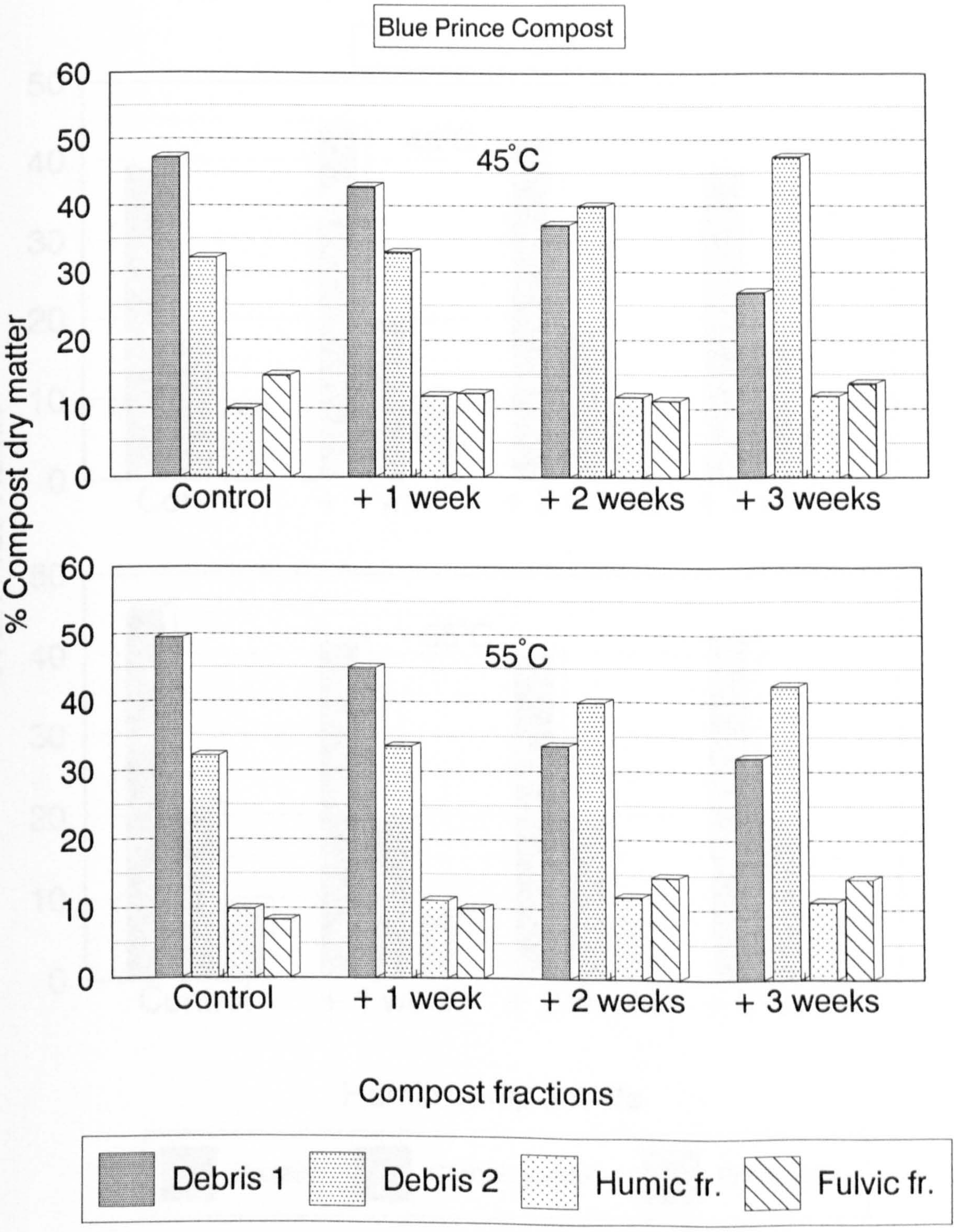


Figure 26. Protein/Carbohydrate/Phenolic content of Ash-Free Humic Material

(Experiment 9 - compost prolonged at 45°C and 55°C)

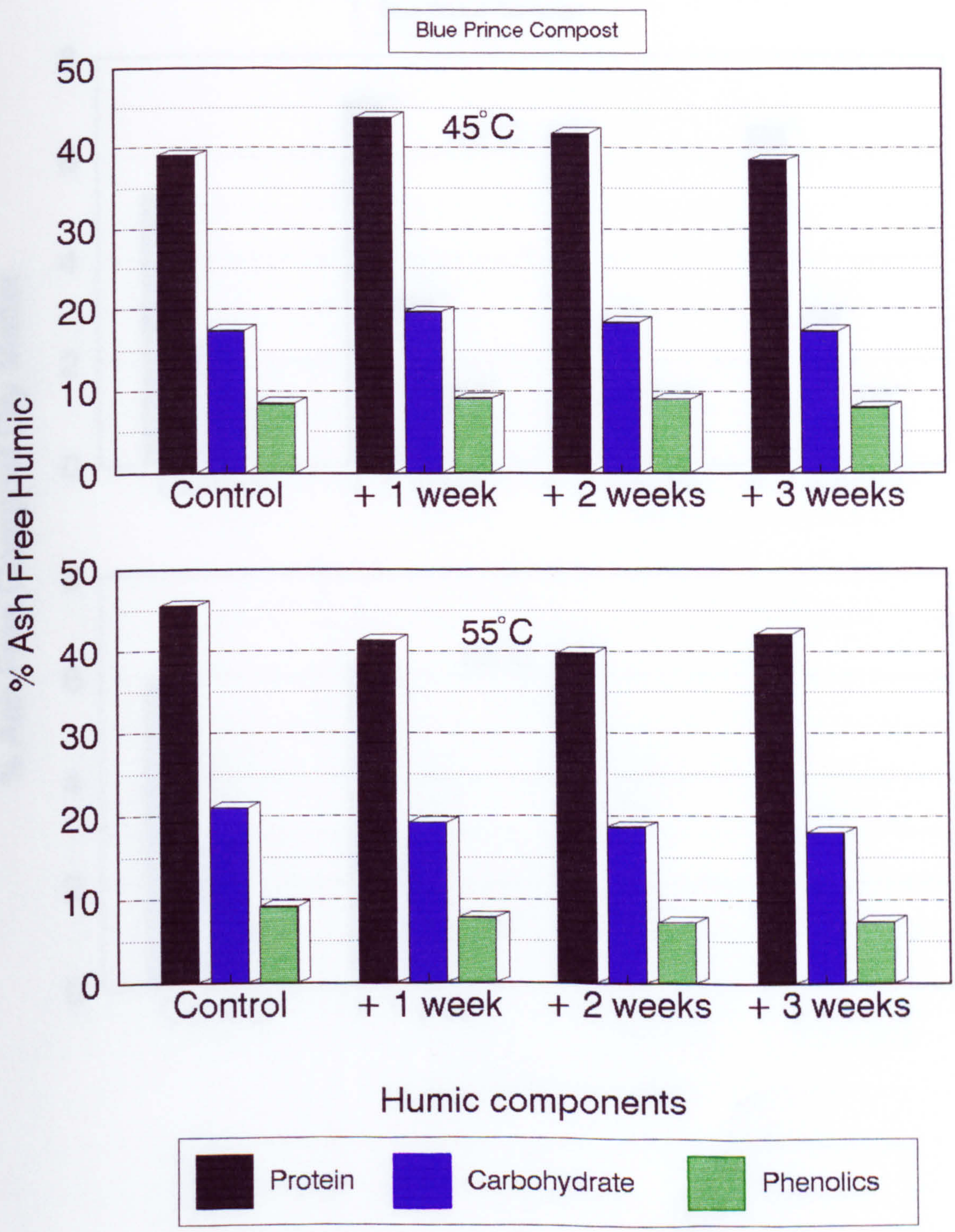


Figure 27. Protein/Carbohydrate/Phenolic content of Humic Fraction

(as a %age of ash-free compost dry matter)

(Experiment 9 - composts prolonged at 45°C and 55°C)

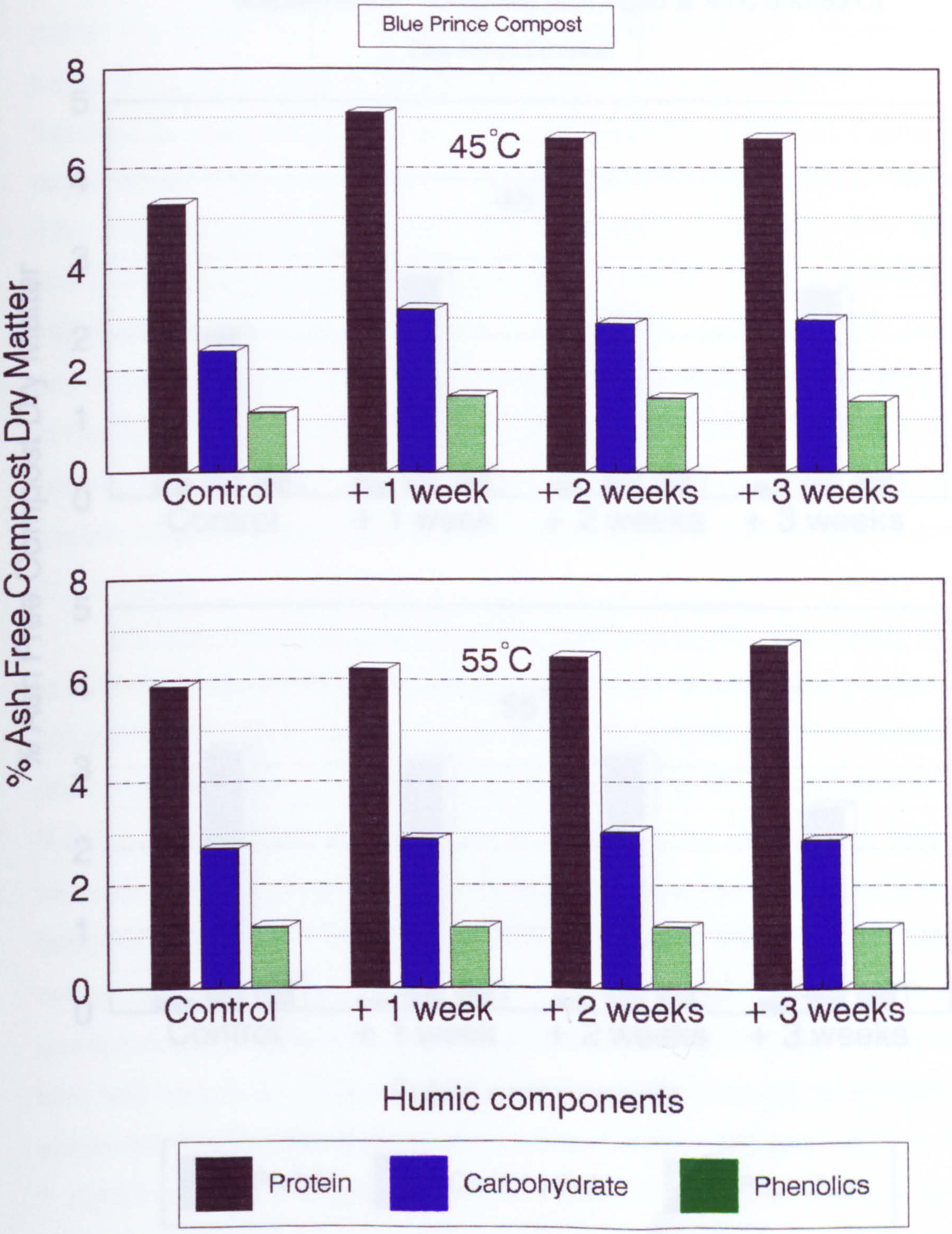
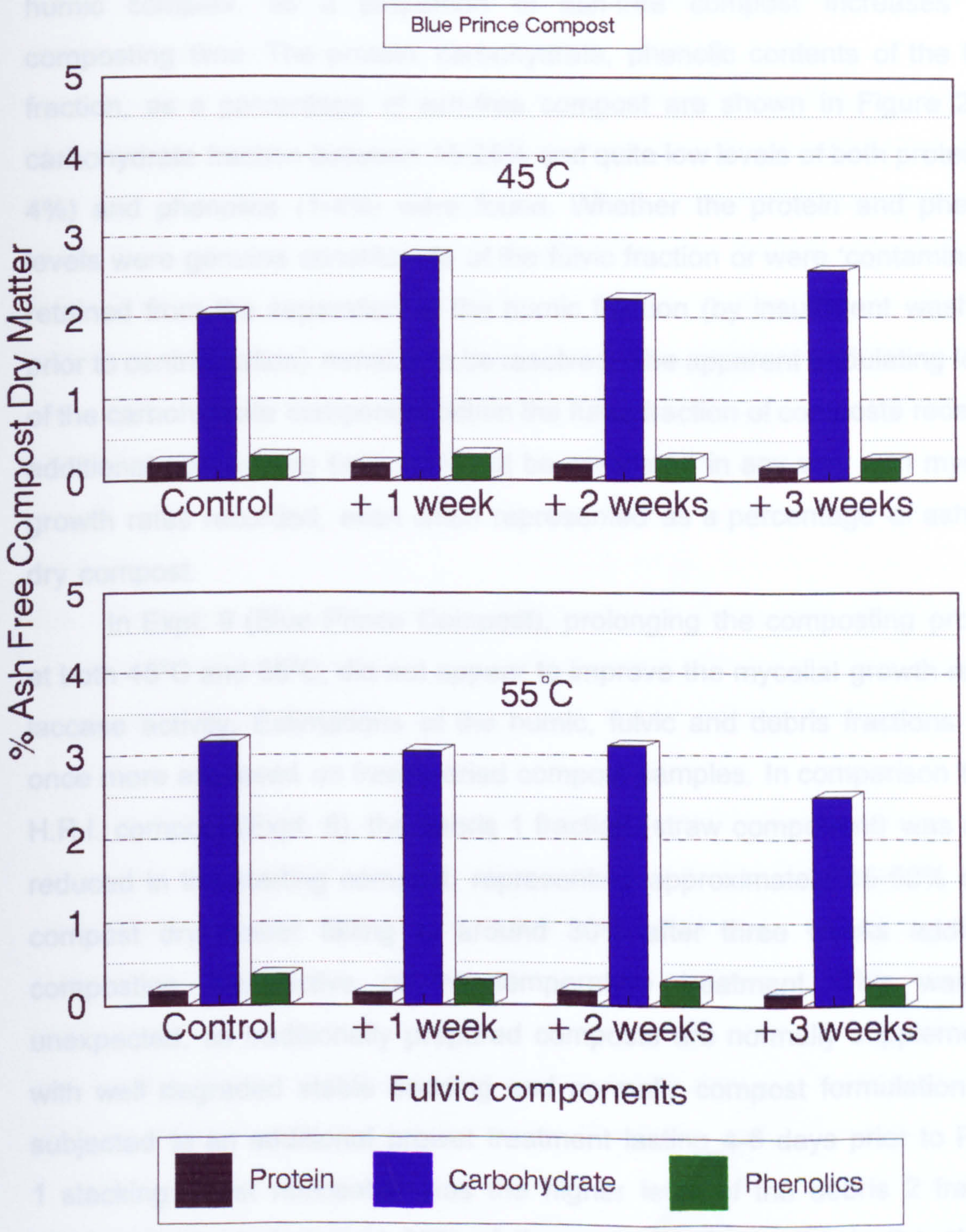


Figure 28. Protein/Carbohydrate/Phenolic content of Fulvic Fraction

(as a %age of ash-free compost dry matter)

(Experiment 9 - composts prolonged at 45°C and 55°C)



mycelial establishment. As prolonging the composting period increases the total ash content of the compost, as organic matter is consumed by the resident microflora, a more meaningful way of presenting the data is to represent the quantities of protein, carbohydrate and phenolics found in the humic fraction as a percentage of the total ash free compost dry matter. This is shown in Figure 23, and it is quite clear that the protein content of the humic complex, as a proportion of ash-free compost increases with composting time. The protein, carbohydrate, phenolic contents of the fulvic fraction, as a percentage of ash-free compost are shown in Figure 24. A carbohydrate fraction between 15-25% and quite low levels of both protein (1-4%) and phenolics (1-4%) were found. Whether the protein and phenolic levels were genuine constituents of the fulvic fraction or were 'contaminants' retained from the separation of the humic fraction (by insufficient washings prior to centrifugation) remains to be resolved. The apparent undulating levels of the carbohydrate component within the fulvic fraction of composts receiving additional composting time could not be correlated in any way with mycelial growth rates recorded, even when represented as a percentage of ash-free dry compost.

In Expt. 9 (Blue Prince Compost), prolonging the composting process at both 45°C and 55°C, did not appear to improve the mycelial growth rate or laccase activity. Estimations of the humic, fulvic and debris fractions were once more assessed on freeze dried compost samples. In comparison to the H.R.I. compost (Expt. 8), the debris 1 fraction (straw component) was much reduced in the starting compost, representing approximately 45-50% of the compost dry matter falling to around 30% after three weeks additional composting irrespective of the temperature treatment. This was not unexpected, as traditionally prepared composts are normally supplemented with well degraded stable bedding and normally compost formulations are subjected to an additional prewet treatment lasting 4-6 days prior to Phase 1 stacking. Most noticeable was the higher level of the debris 2 fraction, which represented around 30% of the compost dry matter in the starting compost rising to levels in excess of 40% after three weeks additional

composting. Surprisingly the humic and fulvic fractions were not dissimilar to that recorded in the H.R.I. formulation even though the compost was darker in colour and more degraded (Figure 25). Analyses of the humic and fulvic fractions for protein, carbohydrate and phenolic content (Figures 26 & 28) were similar to that found in the H.R.I. compost and it was also apparent that the protein content of the humic complex as a proportion of the ash free compost did increase with extended composting time as shown in the previous experiment (Figure 27).

Analysis of the fulvic fraction revealed also that the major component of the fulvic fraction was soluble carbohydrate, possibly overall a little lower than that recorded in the H.R.I. compost when represented as a proportion of ash-free compost dry matter (Figure 28). The main features of experiment 8 and 9 are summarised in Figures 29, 30 and 31, where it is quite clear that ash free humic and the debris 2 fractions steadily increase with composting time, but no correlation could be made between these increases and the mycelial colonisation rates recorded in the two experiments.

4.4 Analyses of 6 Controlled Environment Composts (C.E.C.) with increasing N contents and assessment of humic, fulvic, debris 1 and debris 2 fractions in relation to compost productivity.

Six H.R.I. compost formulations using a combination of fresh wheat straw, deep-litter chicken manure, sporavite (compost activator) and gypsum were prepared with increasing N contents. These formulations were used in a compost programme to prepare selective substrates in a totally controlled environment (bulk pasteurisation tunnels) with little or no Phase 1 composting, Compost samples were taken at the start and at the completion of composting, freeze dried and stored. The proportions of debris 1, debris 2, humic and fulvic fractions are shown in Figure 32. Irrespective of the starting N content of composts, the humic acid component increased very little during the 7-10 days of composting, the most notable increases being

recorded in the debris 2 fractions.

The analytical data of the 6 compost formulations are also presented in Figure 32. For compost formulation 3, two bulk pasteurisation tunnels were used resulting in composts 3(a) and 3(b) with slightly different nitrogen contents. Composting was terminated normally after 6-8 days when the volatile ammonia was reduced to below 10 ppm and this was reflected by the narrow pH range between 7.4 & 7.8 being recorded for this compost series. With compost 6, these conditions were only achieved after 12 days, the additional aeration having a deleterious effect on the compost moisture which was reduced from above 70% to 53.5% which also encouraged the proliferation of sporulating actinomycetes. Only two composts failed to give a mushroom crop and those were the formulations with the highest starting nitrogen and the highest ammonium ion concentration. It is interesting to compare composts 4 & 5 which have similar debris 1, debris 2, humic and fulvic acid profiles at the commencement and completion of composting. The most noticeable difference between the two compost types was the low concentration of the ammonium ion recorded in compost 4 (a highly productive substrate) compared to the level found in compost 5 (very little mushroom colonisation/weed mould establishment). In all other prepared composts i.e. 1, 2, 3(a) & 3(b), reasonable mushroom crops were produced and ammonium levels were close to or below 0.1% of the compost dry matter.

4.5 Nitrogen Balance in debris 1, debris 2, humic and fulvic fractions in high and low N compost formulations at the commencement and completion of composting.

It is now well established (Burrows, 1951a; Smith & Spencer, 1976; Smith, 1977) that the utilisation or conversion of soluble N forms into insoluble forms less readily available is an essential part of producing a selective medium on which *Agaricus bisporus* will grow. To gain a clearer understanding of the distribution of available nitrogen within the compost,

nitrogen analyses were performed on debris 1, debris 2, humic and fulvic fractions at the start and at the completion of composting. For this exercise, two compost formulations were chosen, one with a low N content and one with a very high N content to compare the changes occuring within the isolated fractions. As the analysis was performed on relatively small starting samples and with only 2 replicates, it was decided to perform the procedure a number of times to test the consistency of the results and the pooled data is presented in Tables 15, 16, 17 & 18.

Tables 15 & 16 present the data gathered from 3 separate extraction procedures on a high N compost formulation. While a reasonable consistency of results was apparent for the starting, debris 1 and debris 2 fractions, there was some variation in the humic and fulvic fractions. This could be possibly be explained by the efficiency of material extraction with sodium hydroxide on the day of sonication. Nevertheless, when the results were pooled and the nitrogen levels determined for all 4 fractions, a reasonable balance could be drawn up with the total nitrogen calculated in the starting sample. It was quite clear that the greatest proportion of nitrogen at the commencement of composting was found in the fulvic extract (62%) but this was not surprising considering the amount of deep litter chicken manure included in the formulation to raise the nitrogen level to beyond 3% of compost dry matter. The debris 1 or straw fraction accounted for 25% of the total nitrogen while the humic fraction and debris 2 fractions accounted for 11% and 2.4% respectively. After two phases of composting there were significant increases in the nitrogen within both the debris 2 and humic fractions and reductions within the debris 1 and fulvic fractions. The nitrogen within the debris 2 fraction increased from 2.4% to 20.3% (a nine-fold increase), while the nitrogen content of the humic fraction increased from 10.6% to 19.8% (a two-fold increase). Nitrogen within the debris 1 and fulvic fractions fell to 14.5% and 45.3% respectively.

The analyses of the low nitrogen compost (2 separate extractions) is pooled and presented in Tables 17 & 18. In this compost formulation, a much lower proportion of the total nitrogen can be attributed to the fulvic fraction at

Table 15. Nitrogen Balance of Debris 1, Debris 2, Humic and Fulvic fractions in a high nitrogen compost formulation at the commencement of composting (3 separate analyses combined).

Sep. Analyses.	Compost (total)	Debris 1	Debris 2	Humic	Fulvic
Wt.remaining (g) **N content (% D.M.)**	0.5000 **3.95**	0.3001 **1.52**	0.0216 **2.26**	0.0282 **5.25**	0.1501 **5.10**
Wt.remaining (g) **N content (% D.M.)**	0.5000 **3.42**	0.2978 **1.46**	0.0249 **2.01**	0.0349 **5.89**	0.1424 **7.86**
Wt remaining (g) **N content (% D.M.)**	0.5000 **3.40**	0.3110 **1.42**	0.0225 **1.98**	0.0330 **7.73**	0.1335 **12.06**

Combined analyses	Compost (total)	Debris 1	Debris 2	Humic	Fulvic
Wt. remaining (g) **N content (% D.M.)**	0.5000 **3.59**	0.3029 **1.46**	0.0230 **2.08**	0.0320 **6.29**	0.1420 **8.34**
Wt.of N within fraction	0.01795	0.00468 **(24.6%)**	0.00047 **(2.4%)**	0.00201 **(10.6%)**	0.01184 **(62.3%)**
Summation of all 4 fractions 0.0190					

Table 16. Nitrogen Balance of Debris 1, Debris 2, Humic and Fulvic fractions in a high nitrogen compost formulation at the completion of Phase 2 composting (3 separate analyses combined).

Sep. analyses.	Compost (total)	Debris 1	Debris 2	Humic	Fulvic
Wt. remaining (g) **N content (% D.M.)**	0.5000 **3.33**	0.2394 **1.24**	0.0945 **3.82**	0.0374 **7.42**	0.1287 **7.94**
Wt. Remaining (g) **N content (% D.M.)**	0.5000 **2.95**	0.2256 **1.19**	0.1145 **3.72**	0.0438 **10.70**	0.1161 **7.67**
Wt. Remaining (g) **N content (% D.M.)**	0.5000 **3.02**	0.2320 **1.19**	0.1068 **3.61**	0.0423 **8.59**	0.1116 **6.13**

Combined analyses	Compost (total)	Debris 1	Debris 2	Humic	Fulvic
Wt. remaining (g) **N content (% D.M.)**	0.5000 **3.10**	0.2320 **1.19**	0.1068 **3.61**	0.0423 **8.90**	0.1188 **7.24**
Wt. of N within fraction	0.01550	0.00276 **(14.5%)**	0.00385 **(20.3%)**	0.00376 **(19.8%)**	0.00860 **(45.3%)**
Summation of all 4 fractions 0.01897					

* Figures in parenthesis represent percentage of total nitrogen summation

Table 17. Nitrogen Balance of Debris 1, Debris 2, Humic and Fulvic fractions in a low nitrogen compost formulation at the commencement of composting (2 separate analyses combined).

Sep. Analyses.	Compost (total)	Debris I	Debris II	Humic	Fulvic
Wt.remaining (g) **N content (% D.M.)**	0.5000 **1.47**	0.3716 **0.67**	0.0143 **2.01**	0.0171 **3.53**	0.0954 **2.84**
Wt.remaining (g) **N content (% D.M.)**	0.5000 **1.51**	0.3746 **0.92**	0.0143 **2.06**	0.0185 **3.35**	0.0937 **2.78**

Combined analyses	Compost (total)	Debris I	Debris II	Humic	Fulvic
Wt. remaining (g) **N content (% D.M.)**	0.5000 **1.49**	0.3731 **0.80**	0.0143 **2.04**	0.0178 **3.44**	0.0946 **2.81**
Wt.of N within fraction	0.00745	0.00298 **(46.6%)**	0.00029 **(4.4%)**	0.00061 **(9.3%)**	0.00265 **(40.6%)**
Summation of all 4 fractions 0.00653					

Table 18. Nitrogen Balance of Debris 1, Debris 2, Humic and Fulvic fractions in a low nitrogen compost formulation at the completion of Phase 2 composting (2 separate analyses combined).

Sep. analyses.	Compost (total)	Debris I	Debris II	Humic	Fulvic
Wt. remaining (g) **N content (% D.M.)**	0.5000 **1.88**	0.3024 **0.72**	0.0542 **2.34**	0.0438 **5.43**	0.1018 **3.22**
Wt. Remaining (g) **N content (% D.M.)**	0.5000 **1.87**	0.3115 **0.80**	0.0525 **2.60**	0.0387 **6.77**	0.0972 **4.02**

Combined analyses	Compost (total)	Debris I	Debris II	Humic	Fulvic
Wt. remaining (g) **N content (% D.M.)**	0.5000 **1.875**	0.3070 **0.76**	0.0534 **2.47**	0.413 **6.10**	0.0995 **3.62**
Wt. of N within fraction	0.00944	0.00233 **(23.8%)**	0.00132 **(13.5%)**	0.00252 **(25.8%)**	0.00360 **(36.9%)**
Summation of all 4 fractions 0.00977					

* Figures in parenthesis represent percentage of total nitrogen summation

the commencement of composting. As with the high nitrogen compost, there were significant increases in nitrogen content of both the debris 2 and humic fraction during composting, a significant decrease in the debris 1 fraction but only a small reduction in the fulvic fraction.

For both the high and low-nitrogen formulated composts the summation of the nitrogen present in the debris 1 and fulvic fractions at the commencement of composting amounted to 86% of the total nitrogen present. At the completion of composting these two fractions when combined had fallen to around 60% of the total nitrogen present.

Similarly if we combine the two fractions that increase in nitrogen content during the composting process i.e. debris 2 and the humic fraction, we can assess that these two fractions contain around 13/14% of the total nitrogen at the start of composting increasing to a level close to 40% at the completion of composting.

4.6 Concluding Comments

The first sonication experiments (S1 & S2) to extract humic substances from freeze dried compost samples were performed on an H.R.I. formulation using freshly harvested wheatstraw. The compost used was very light in colour, which is generally a good indicator of low levels of cellulose/hemicellulose being utilised during the two composting phases and a low microbial biomass (Smith, 1980). The humic and debris 2 fractions were estimated, at approximately 3-7% and 8-11% respectively, much lower than the levels found in the later experiments, where a darker more typical compost was used. The humic acid contents estimated in the compost used in experiment S3 & the repeat experiment S4 were close to 10% whereas the debris 2 fractions were a little above 20% (Figure 19). As an outcome of these preliminary studies, it was decided to use a sonication time of 10 minutes as within this period of time there was little solubilisation of plant polymers irrespective of compost type and the estimated levels of humic substances were reasonably accurate. This was confirmed by electron

microscopy (Plates 6, 7 , 8 , 10 & 11). These clearly demonstrate the efficient removal of the humic layer from the cereal straw surface by sonication of freeze dried compost samples with 0.5M NaOH. The alkali insoluble debris 2, separated by centrifugation at 9,000 rpm, also clearly shows the high level of bacterial and fungal cells contained within this fraction (Plate 9).

Analyses of the humic and debris 2 fraction from the compost used in Expt. 8 confirmed the findings of S3 & S4, levels being close to 10% and 20% of the dry matter respectively. Prolonging the composting phase was also shown to increase the debris 2 fraction, whereas the humic fraction remained comparatively constant at 10% of the compost dry matter. Analysis of the compost taken from a local farm (Blue Prince, Poling, Sussex) and used in Expt. 9 gave some interesting data in that the humic fraction was almost identical to the H.R.I. formulation but the debris 2 fraction was about 30% of the compost dry matter, a 50% increase. Furthermore, while the humic fraction remained reasonably constant with prolonged composting times, the debris 2 fraction increased to beyond 40% of the compost dry matter irrespective of the temperature regime employed (i.e. 45° or 55°C). It was quite clear that when the humic material was analysed in these two compost types that protein contributed upwards of 40% of the humic dry weight, with carbohydrate and phenolic contents being approximately 20% and 10% respectively (Figures 22 & 26). A clearer picture of how humic components (protein, carbohydrate, phenolics) feature in a prepared compost is to represent them as a percentage of ash free compost (Figures 23 & 27). Protein levels contributed to between 4-6% of the dry matter, whereas carbohydrate and phenolic monomers were close to 2% and 1% respectively.

The straw coloured fraction remaining after extraction of humic and debris material, and conveniently called the fulvic fraction was also analysed for protein, carbohydrate and phenolic components. Although there were some inconsistencies between control samples analysed on different days (see Figure 28), it was quite clear that this residual fraction contained upwards of 20% carbohydrate and low levels of protein and phenolic monomers. When these figures are represented as a proportion of ash-free

Figure 29. Ash-Free Humic content of two distinct compost types

(after extended composting periods at 45°C and 55°C)

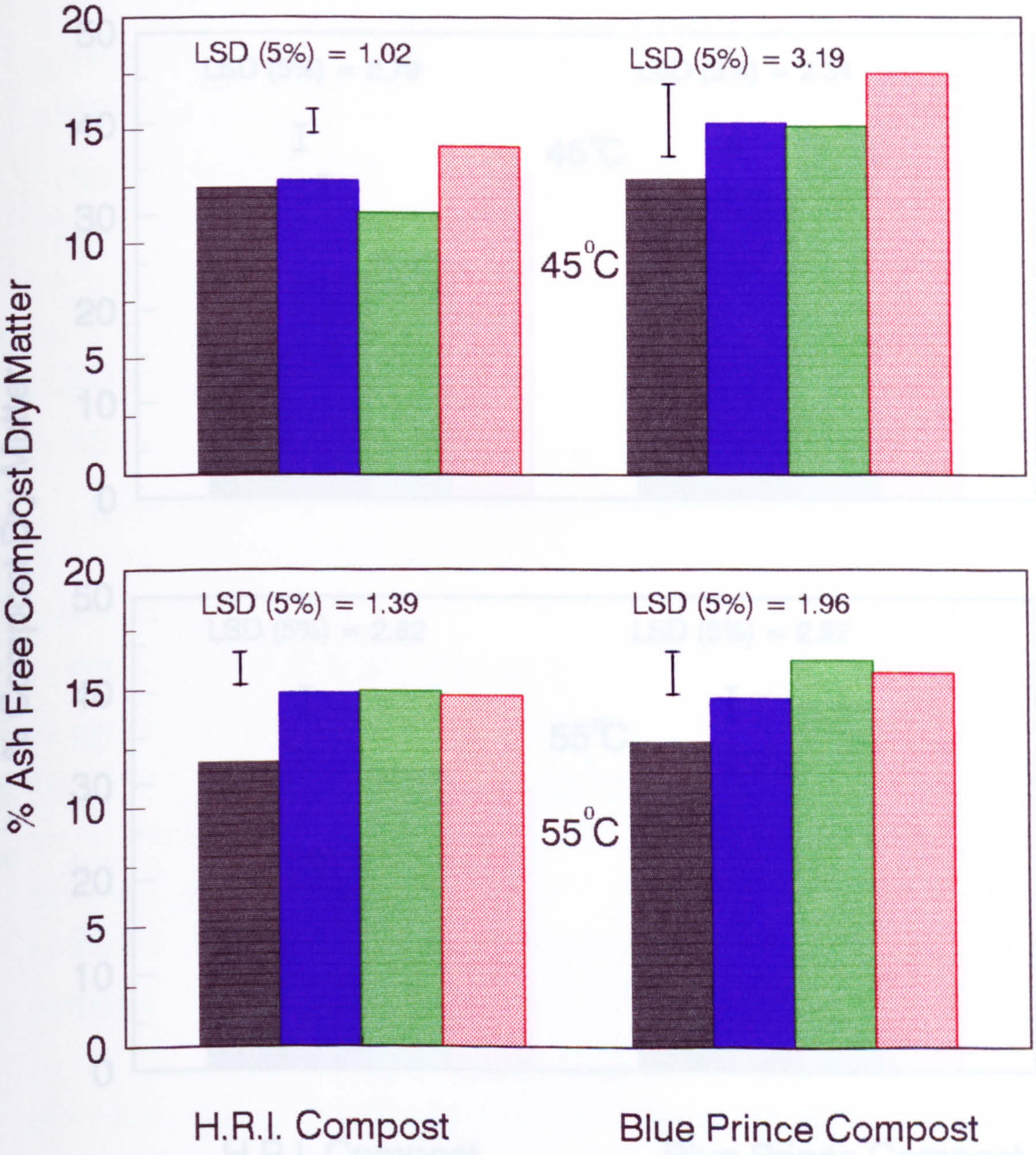


Figure 30. Changes in Debris 2 content (% compost dry matter) of two distinct compost types during extended composting periods

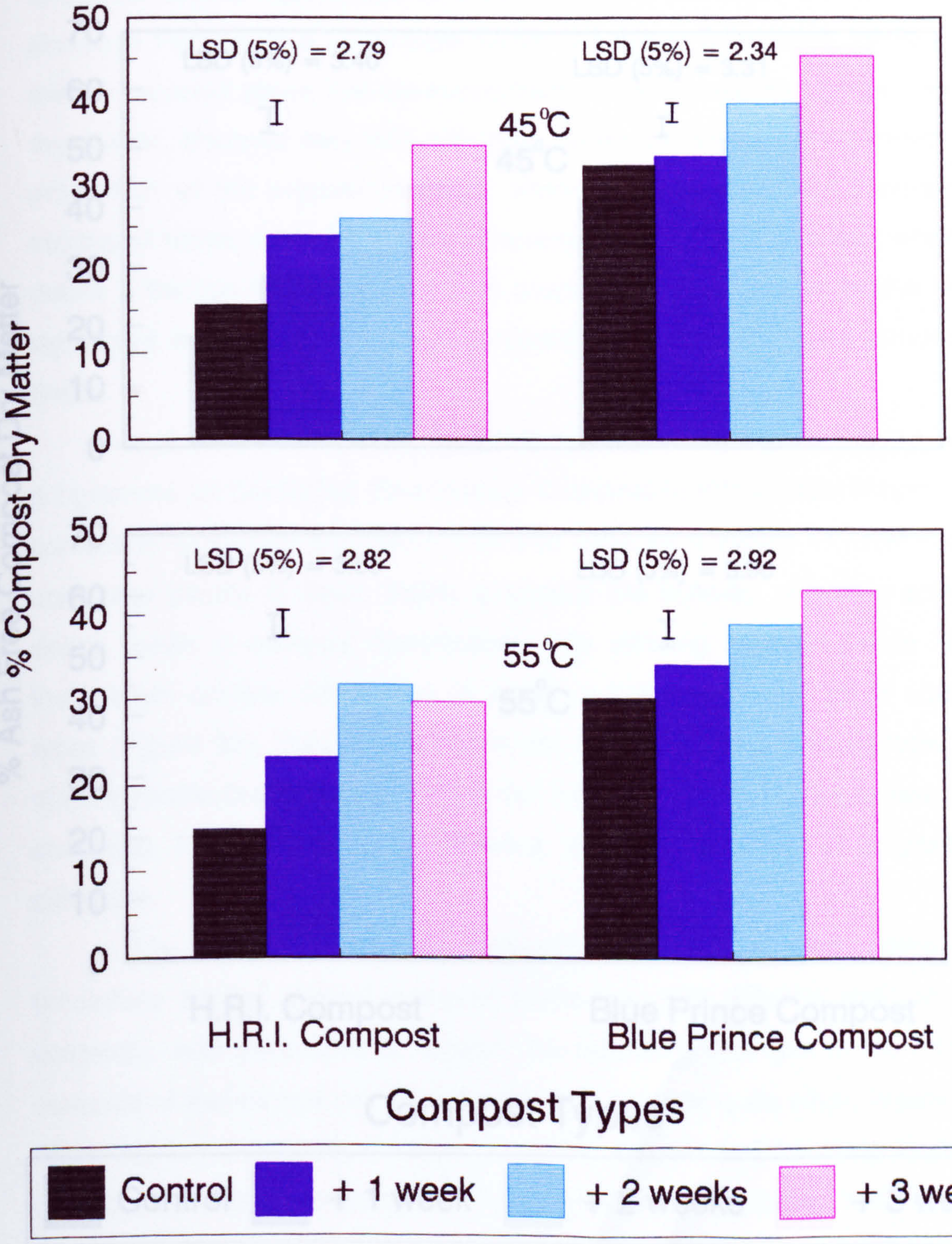
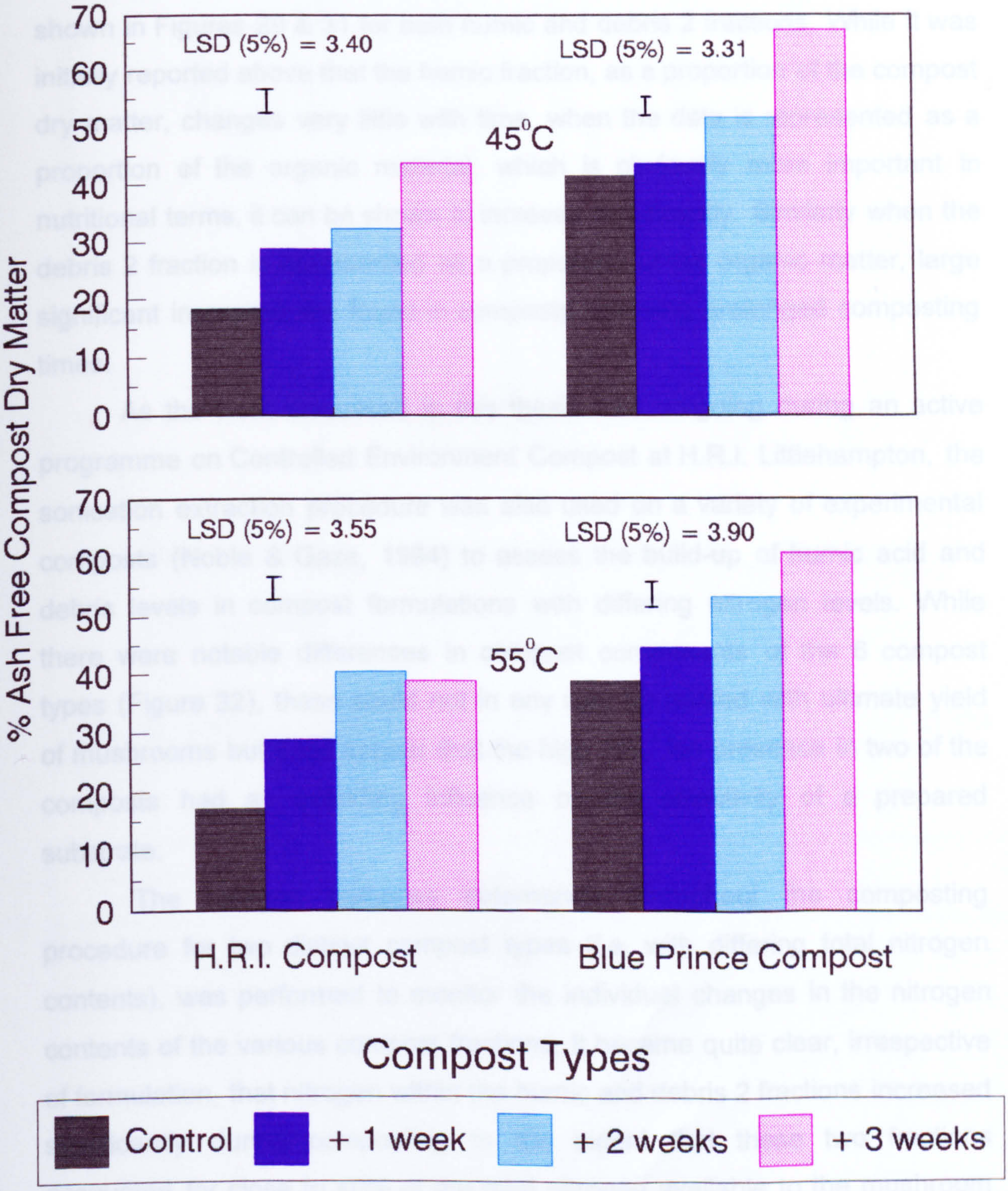


Figure 31. Changes in Debris 2 content (% ash-free compost dry matter) of two distinct compost types during extended composting periods



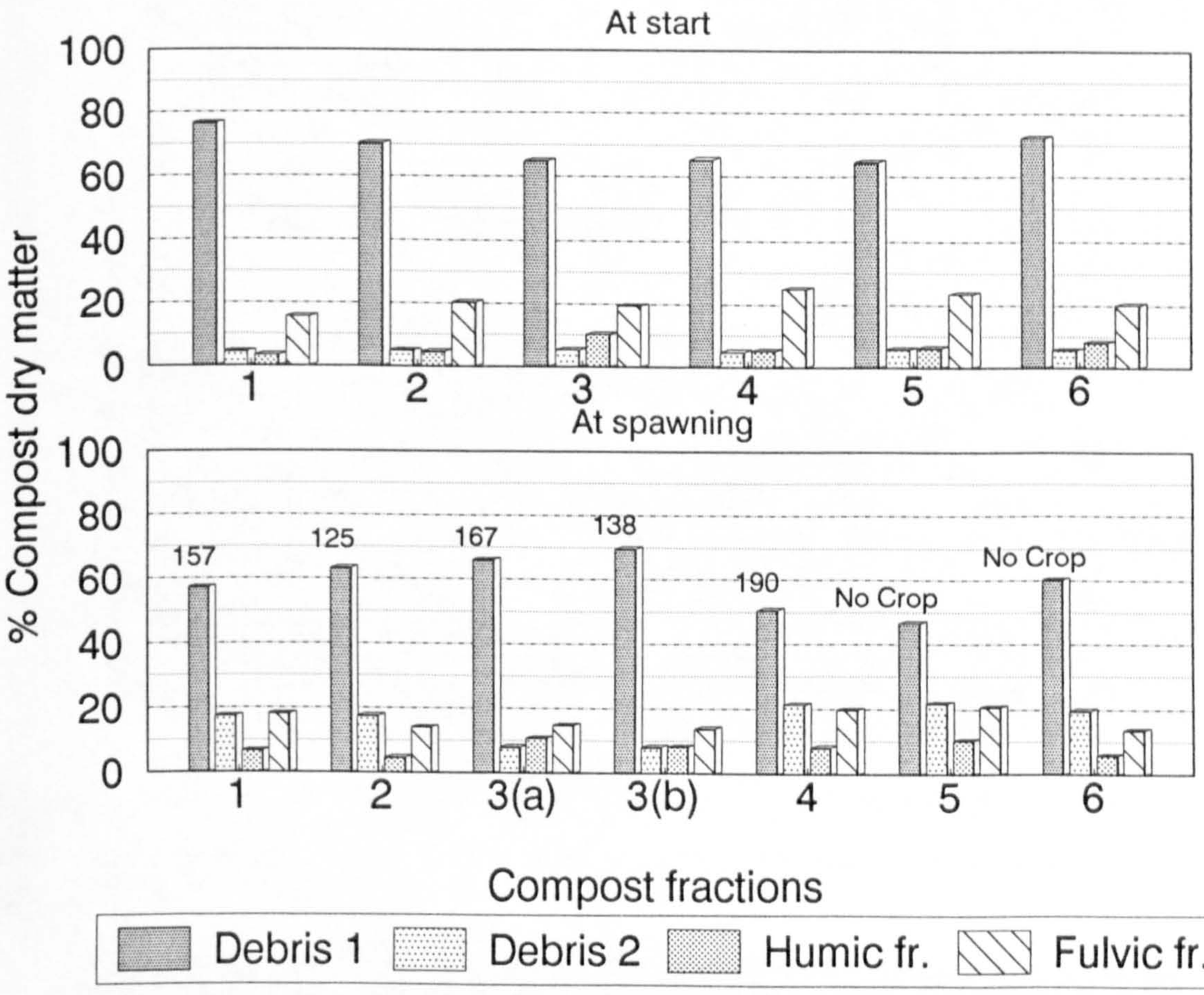
compost dry matter (Figures 24 & 28), it is clear that the carbohydrate component within the fulvic fraction is the major component at levels between 3-4%. The ash content of organic composts increases with time as the cellulose/hemicellulose component is degraded by the resident microflora but a truer picture of changes in compost constituents can be seen if the analytical data is represented as a percentage of the organic matter. This is shown in Figures 29 & 31 for both humic and debris 2 fractions. While it was initially reported above that the humic fraction, as a proportion of the compost dry matter, changes very little with time, when the data is represented as a proportion of the organic material, which is obviously more important in nutritional terms, it can be shown to increase significantly. Similarly when the debris 2 fraction is represented as a proportion of the organic matter, large significant increases are found in composts receiving prolonged composting times.

As the work discussed in this thesis was on-going during an active programme on Controlled Environment Compost at H.R.I. Littlehampton, the sonication extraction procedure was also used on a variety of experimental composts (Noble & Gaze, 1994) to assess the build-up of humic acid and debris levels in compost formulations with differing nitrogen levels. While there were notable differences in compost components of the 6 compost types (Figure 32), these could not in any way be related with ultimate yield of mushrooms but it did appear that the high NH_4^+ ion presence in two of the composts had an overiding influence on the selectivity of a prepared substrate.

The nitrogen balances determined throughout the composting procedure for two distinct compost types (i.e. with differing total nitrogen contents), was performed to monitor the individual changes in the nitrogen contents of the various compost fractions. It became quite clear, irrespective of formulation, that nitrogen within the humic and debris 2 fractions increased significantly during composting to the extent that these two fractions accounted for close to 40% of the total nitrogen available to the mushroom at spawning. These increases were offset by reductions in the nitrogen content of both the straw (debris 1) and fulvic fractions.

Figure 32. Compost Components of 6 "Controlled Environment" Composts

at the start and completion of two phases of composting



Numbers indicate yield of mushrooms [kg/tonne compost at spawning]

Table 19. Analyses of Controlled Environment Composts (1-6) at the start and completion of composting.

Compost No.	1.	2.	3(a).	3(b).	4.	5.	6.
N (st)	1.13	1.58	1.61	1.61	1.85	2.40	2.75
N (sp)	1.64	1.87	2.11	1.71	2.15	2.55	3.29
% Moisture	74.0	68.2	68.3	73.1	67.7	64.5	53.5
pH (sp)	7.51	7.41	7.51	7.83	7.46	7.75	7.64
NH_4^+ (sp)	0.03	0.12	0.04	0.11	0.07	0.31	0.28
Yield (Kg/tonne)	157	125	167	138	190	No Crop	No Crop

st = at start; sp = at spawning

ELECTRON MICROSCOPY PLATES

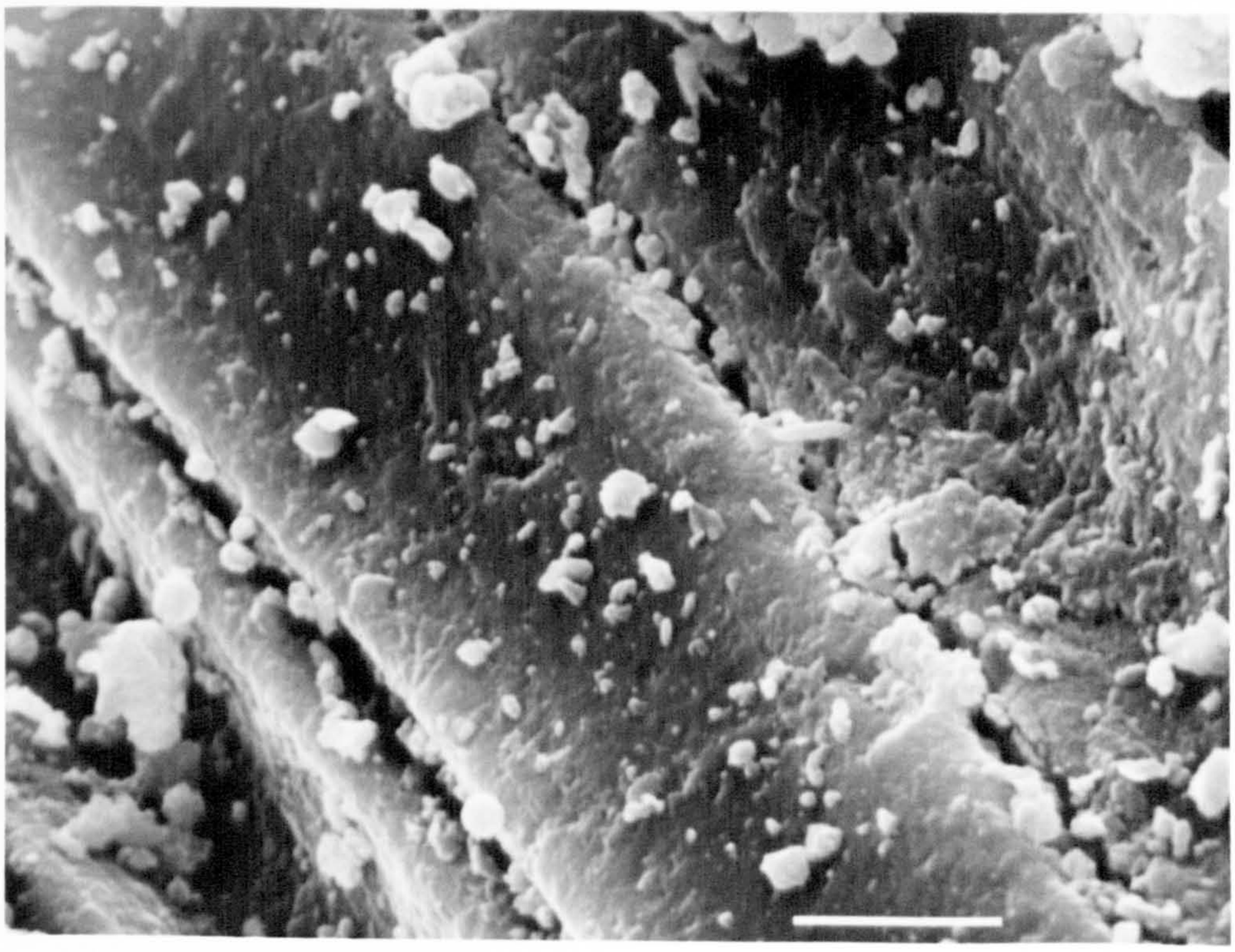


Plate 6. Freeze dried compost sample before sonication (Bar = 5μm). Photograph shows microbial fragments embedded in the humic layer which accumulates on the straw surface during composting.

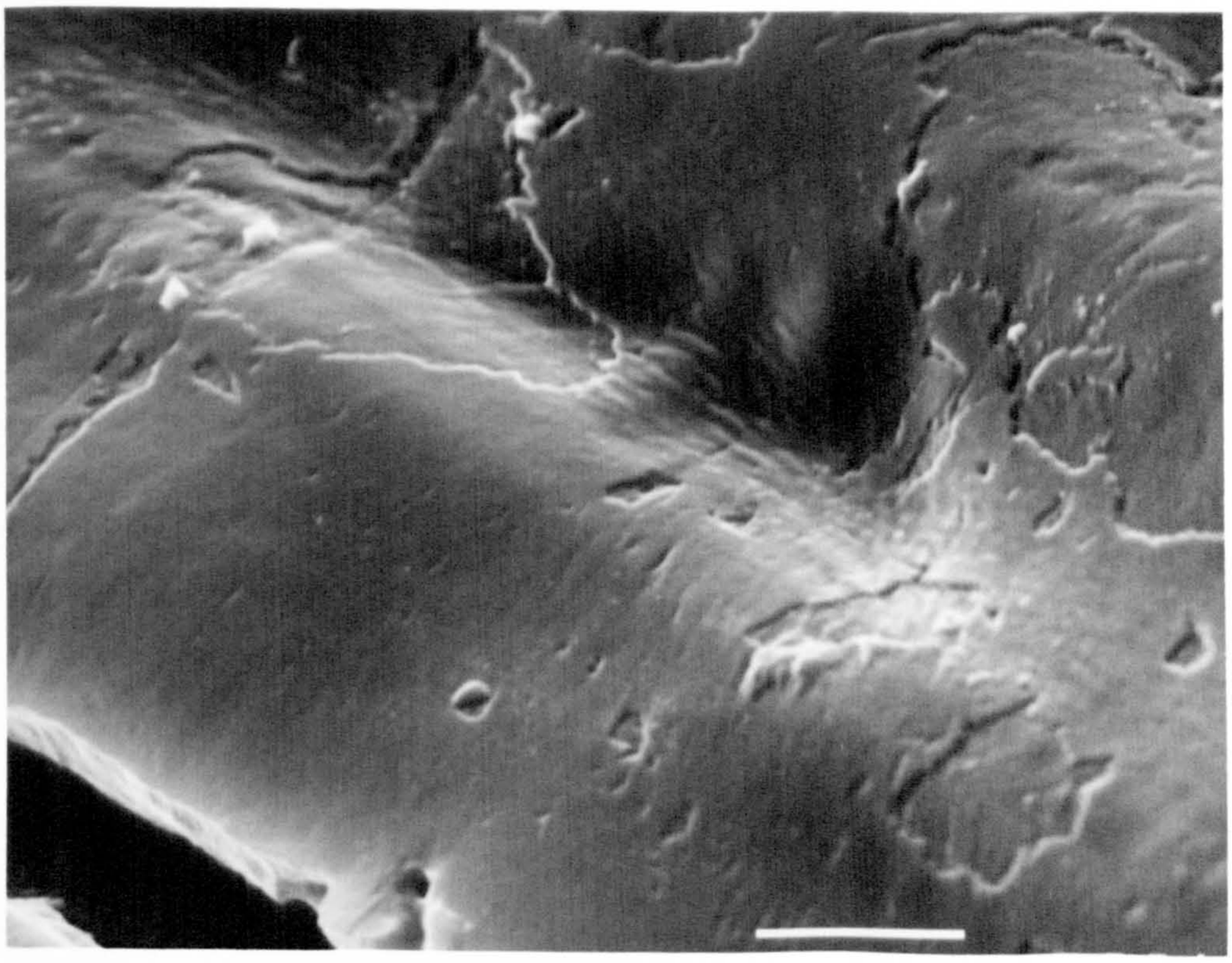


Plate 7. Freeze dried compost sample after 10 minutes sonication with 0.5M NaOH (Bar = 5μm). Photograph shows the straw surface after removal of the humic layer.

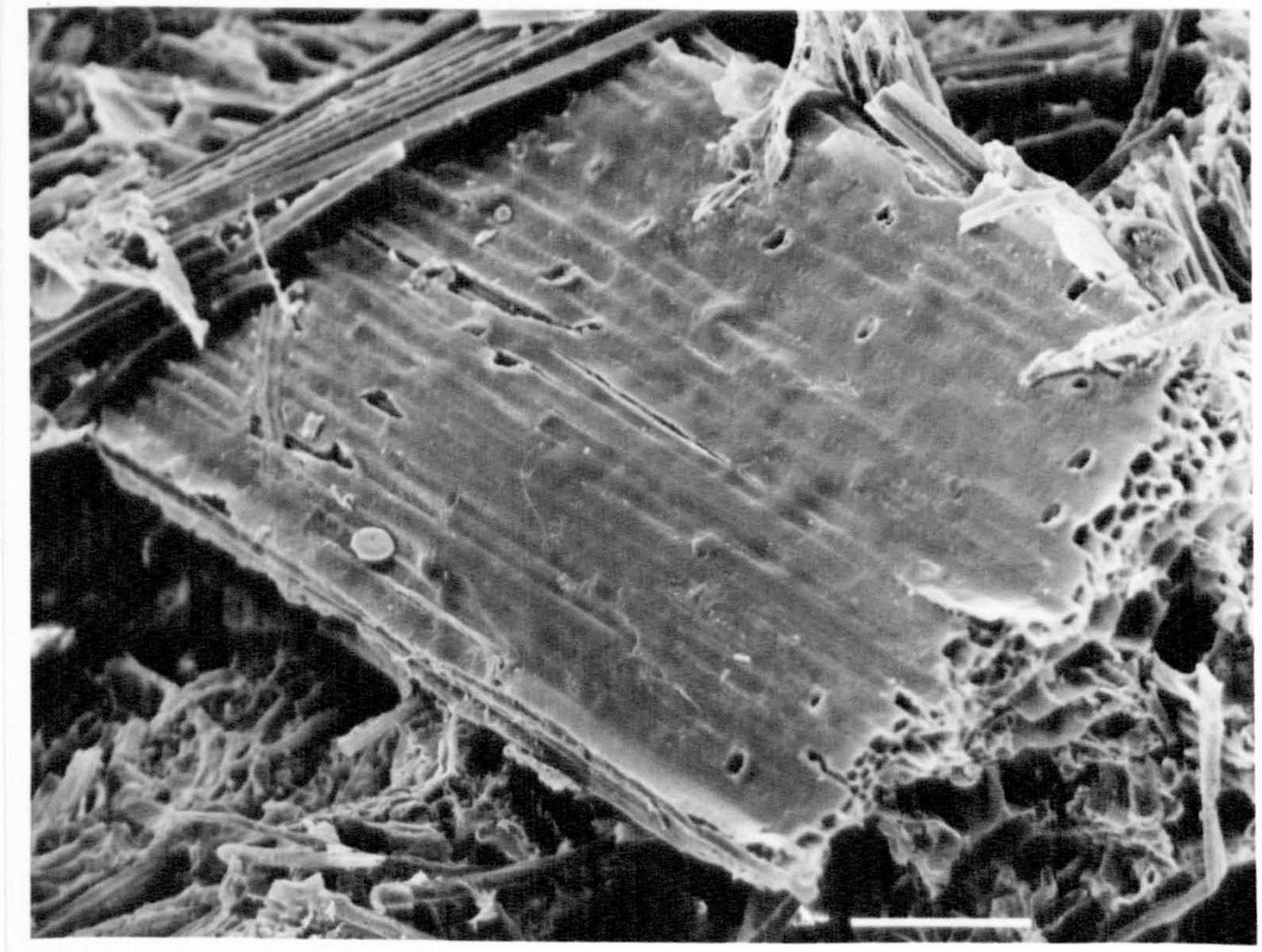


Plate 8. Debris 1 fraction showing straw fragments with humic layer removed (Bar = 100μm).

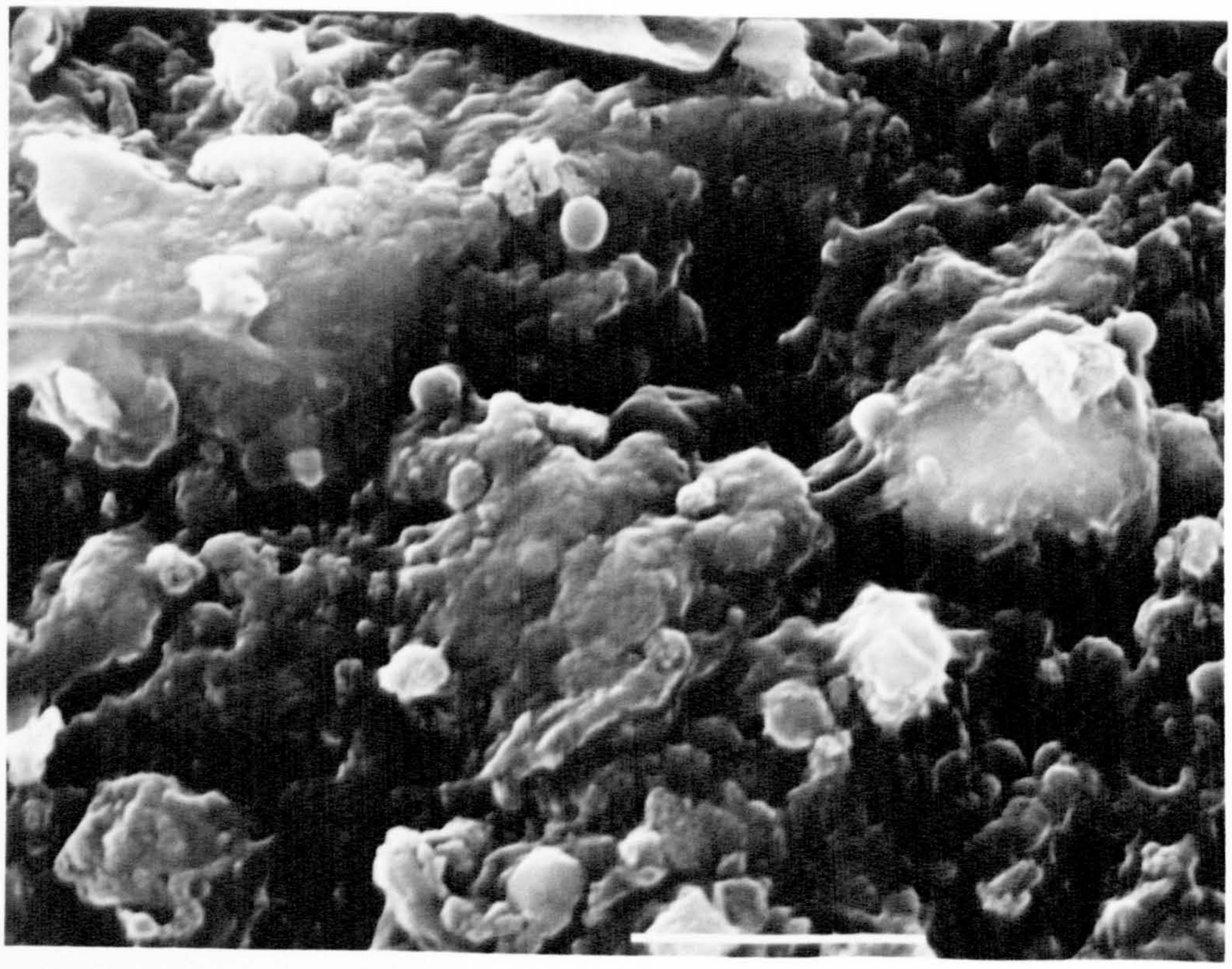


Plate 9. Alkali insoluble debris 2 separated by centrifugation at 9,000 rpm (Bar = 5μm). Photograph emphasises the high proportions of fungal and bacterial fragments contained within this fraction.

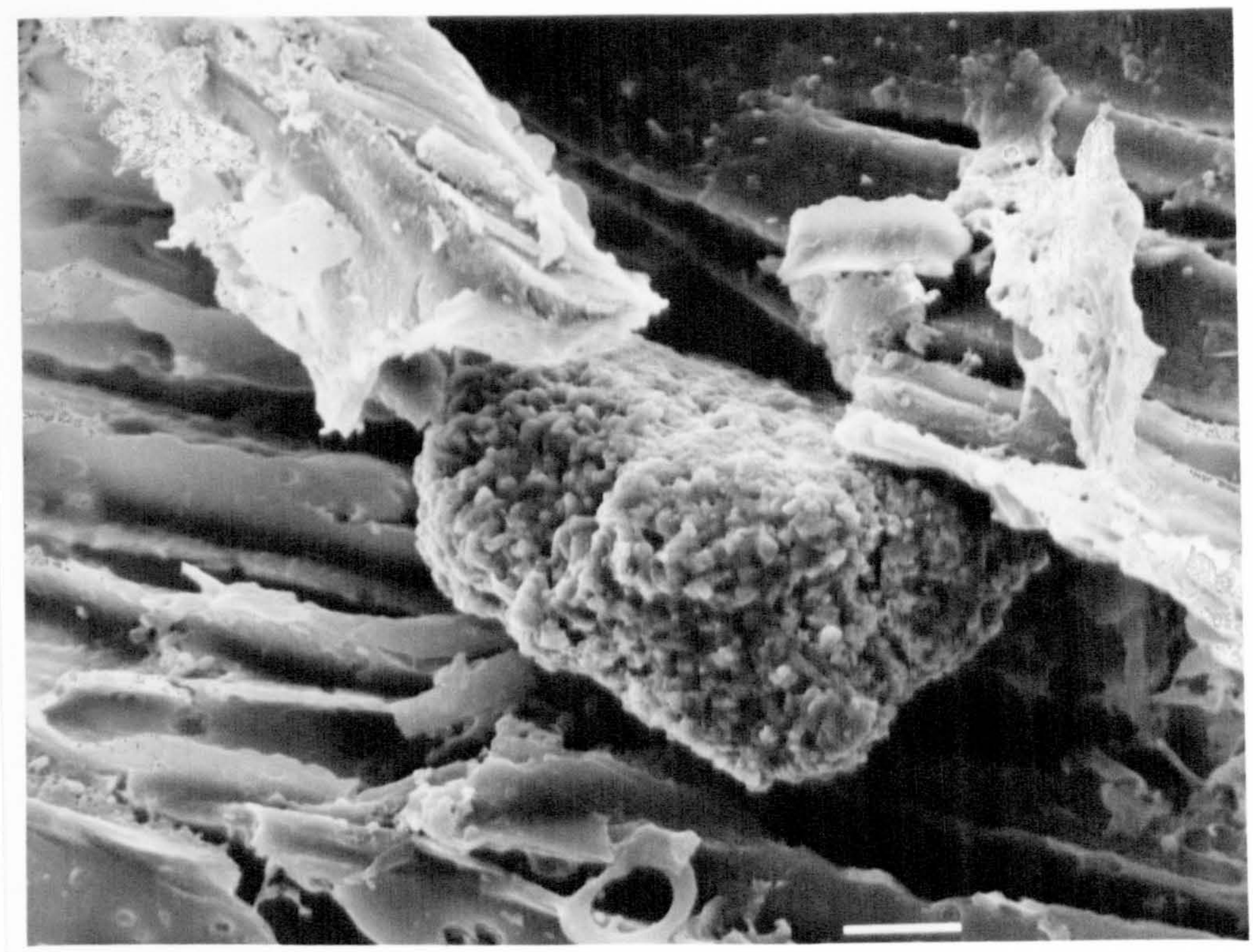


Plate 10. Intact microbial colonies separating from vascular bundle during alkali extraction (Bar = 10μm).

Plate 11. Cross sectional view of cereal straw vascular system after extraction of humic and microbial biomass (Bar = 10μm)

CHAPTER 5 ASSESSMENT OF DEBRIS 1, DEBRIS 2, HUMIC AND FULVIC ACID CHANGES DURING SUBSTRATE COLONISATION

5.1 Small scale investigation using 'race tubes'.

All the analyses so far described in this thesis have been performed on freeze dried compost samples taken throughout composting or at the completion of composting. To gain a clearer understanding of selectivity and the rate at which mushroom mycelium colonises its substrate, a small scale investigation was performed to determine which fractions were the first to be utilised during colonisation. Sufficient 'race tubes' were set up and inoculated with *A. bisporus* (U3) spawn (See 2.7). During a colonisation period of 41 days, 3 replicate tubes were randomly selected after 10, 17, 26 and 41 days, the contents removed, pooled, well mixed and analysed for their debris 1, debris 2, humic and fulvic contents.

At the time of inoculation (Fig. 33), the humic fraction was found to comprise approximately 5% of the compost dry matter and there was to be little evidence of a major change in this during the colonisation period. The debris 2 fraction of this compost significantly increased during composting from approximately 5% of the compost dry matter to over 20% at the time of spawning. Analysis revealed that it remained at this level up until day 17 when its level appreciably dropped. This coincided with the sudden increase in laccase activity. To check whether this was a real effect, the ash-free debris 2 levels were determined and replotted on the same graph. Ash contents were found to be remarkably stable (see Appendix 13), at around 30%, and the ash-free debris 2 component was once again shown to decline after 17 days (Figure 33).

During composting there is a continuous utilisation of organic matter by the compost microorganisms, and consequently the ash content, as a proportion of the remaining compost dry matter increases. However, the amount of ash can be assumed to remain approximately constant at all times.

If the analytical data represented in Figure 34 is then corrected on an equal ash basis a clearer picture of the loss and accumulation of the various fractions emerges (Figure 35). The debris 1 fraction diminishes from well over 60% of the compost dry matter to around 30%, which is to be expected as the cellulose/hemicellulose degradation rate is high during the colonisation by mushroom mycelium. While there would appear to be only small changes in the humic and fulvic fractions, it is quite clear that the decline in the debris 2 fraction in the latter stages of mycelial colonisation is a real one. While changes in these fractions during the composting phase can be accurately determined, changes occurring during substrate colonisation by mushroom mycelium are less accurate because of the further complication of fungal biomass accumulation. This can reach up to 7% of the compost dry matter after 41 days (see appendix 12). Although this amount of material is conveniently superimposed on the debris 1 fraction in Figure 35, it must be remembered that this amount of dry matter, by virtue of the analytical procedure adopted (i.e. milling, sonication, centrifugation and re-precipitation), is actually present in all 4 fractions.

5.2 Comparison of enzyme profiles (laccase, endo-cellulase, β-N-acetyl glucosaminidase and β-N-acetyl muramidase) with substrate depth and association with changes in compost constituents.

During investigations to analyse the lower production efficiency of mushroom substrates with time when contained in deep beds or troughs 1 metre deep (Smith *et al*, 1989), the pattern of endocellulase activity in the uppermost layers of compost was shown to be similar to that found by Claydon *et al* (1988). Peaks of activity coincided with peaks of fruitbody production. Compost samples taken from the lower layers of compost showed a steady decline in endocellulase activity and was barely detectable in the bottom layer. In the uppermost layer, the largest peak of cellulase activity

Figure 33. Changes in the Debris 2 and Humic Fractions of compost in relation to Laccase activity

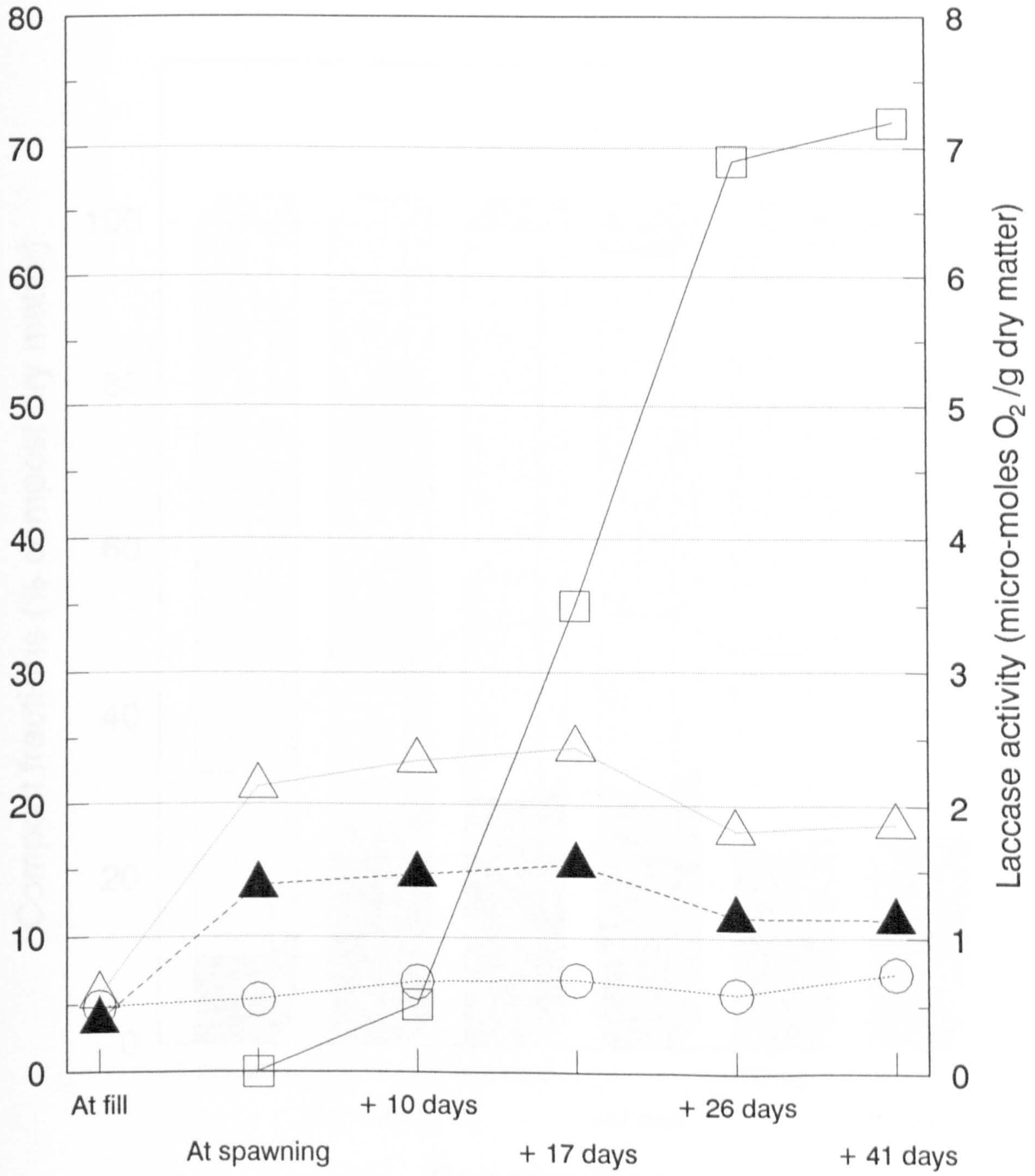


Figure 34. Changes in the proportions of compost fractions during composting and throughout mycelial colonisation

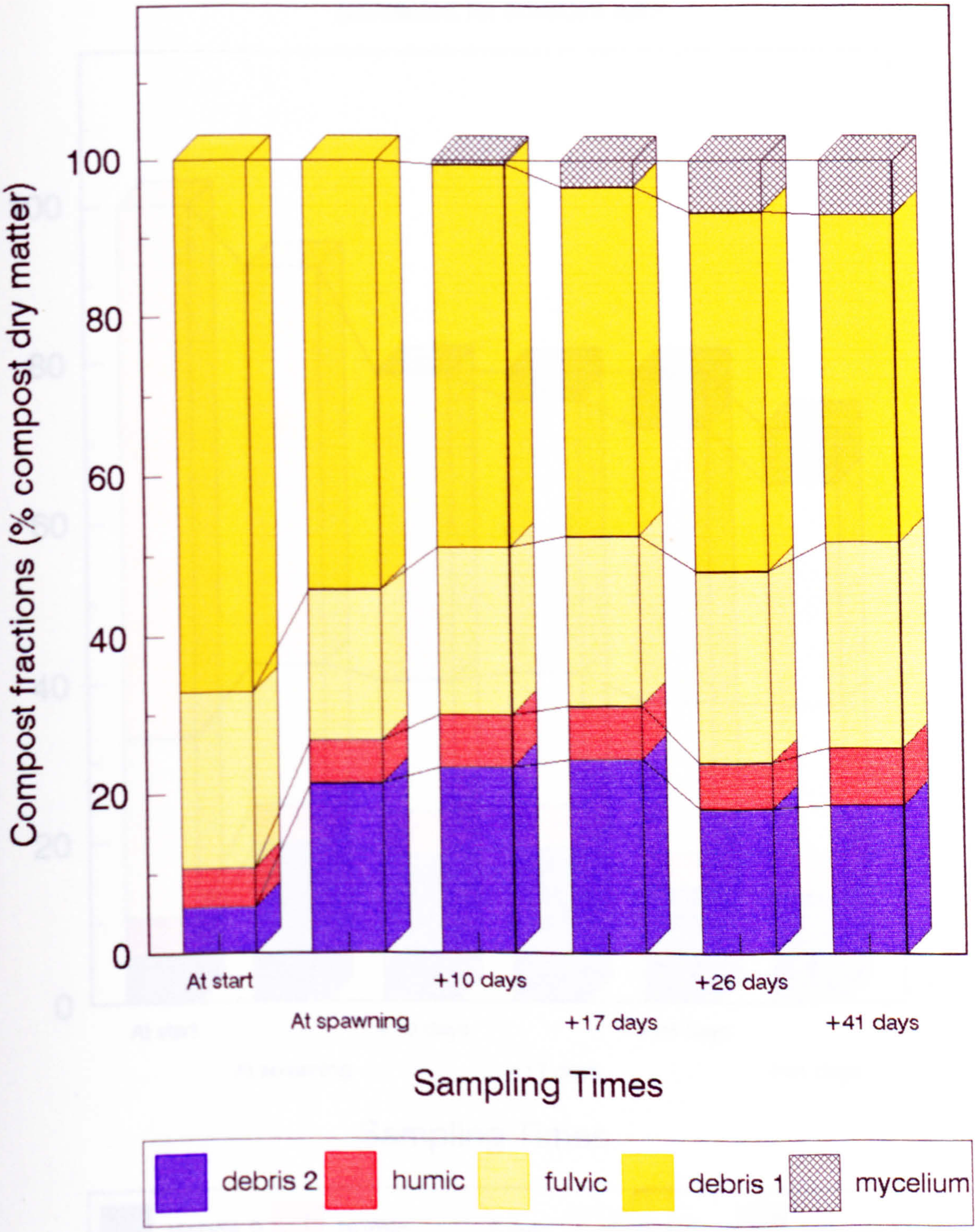


Figure 35. Changes in the proportions of compost fractions during composting and throughout mycelial colonisation

(corrected for constant ash)

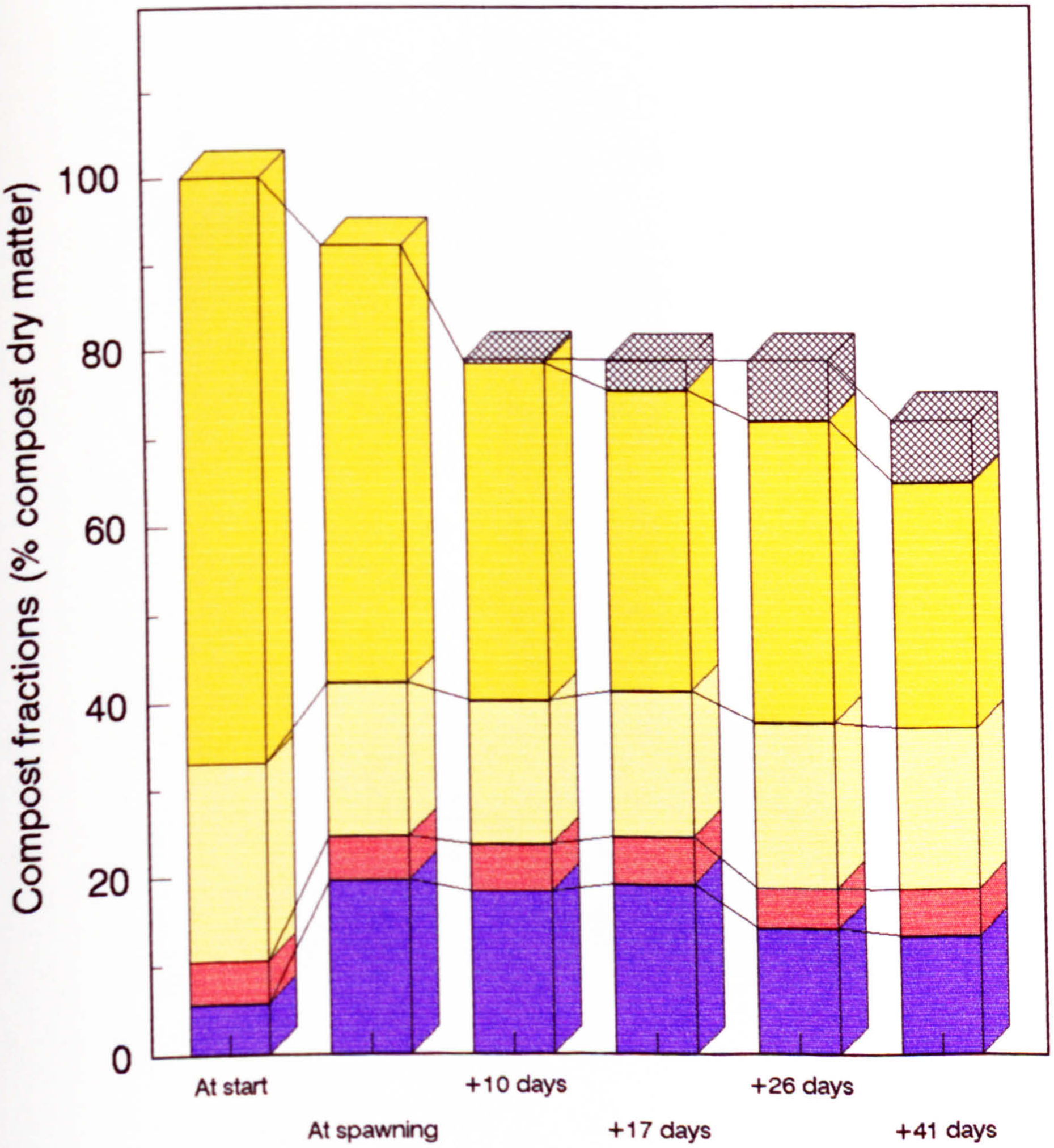


Plate 12. Compost column (1.5m high) divided into 5 separate compartments (top, upper middle, middle, lower middle and bottom) to facilitate compost sampling.

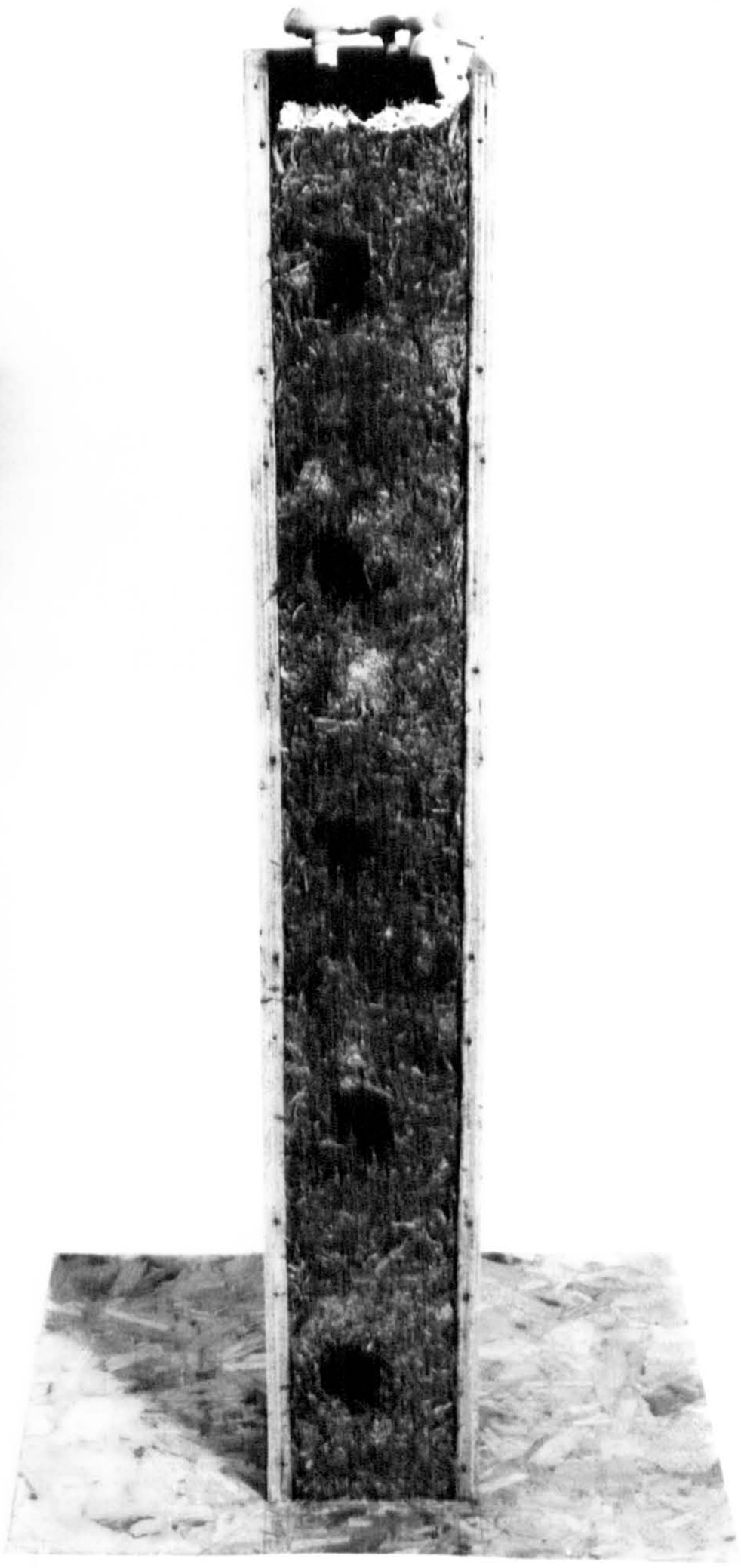


Plate 13. Compost column with side panels removed to show compost sampling points. N.b. compost sampled initially on left hand side; further samples taken to the right.

occurred at the onset of the first flush. In the middle layer, 0.5 metres down from the fruiting surface, there was a gradual increase in cellulase activity from the first to second flush increasing substantially during the third flush. At around 0.75 metres down, the onset of elevated cellulase activity was delayed until the fifth flush.

Laccase activity produced by the mushroom mycelium was also assayed throughout the deep bed. Laccase, thought to be associated with detoxification of phenolic complexes, increased and remained at a high level until the appearance of mushrooms, when it declined rapidly. Unlike cellulase activity, which decreased steadily with depth, laccase activity increased. In the lower layers, very high laccase levels were recorded and were sustained for long periods. The role of laccase activity in *A.bisporus* growth and development is still uncertain but such enzyme activity may be required for detoxification or degradation of phenolic complexes accumulated during the composting process (Wood & Smith, 1986) prior to carbohydrate utilisation.

The modulation in enzyme activities in a deep substrate culture was consistent with the data of Smith (1984) who showed a progressive downward utilisation of the solid substrate nutrients with successive cycles of fruitbody production. It was concluded that the poor production efficiency from deep beds of compost was due to the distance nutrients have to travel to the fruiting surface. This would explain why shallow layers of compost are the most productive.

As a continuation of these studies, two identical elongated boxes or columns (Plate 12) were constructed (See 2.10) to study how differing enzyme profiles affected the utilisation of all compost fractions in cased and uncased composts. Compost samples were cut out centrally, on the left side from each of the five sections (Plate 13). Further samples, taken at a later date, were then cut to the right of the first sample, to leave an uninterupted mycelial network.

Laccase levels found in the uppermost layer of the column confirmed the earlier findings of Wood & Goodenough (1977) with a rapid decline at the onset of fructification. This decline was less apparent in the lower sections

(Figure 37). After two flushes of mushrooms (+25 and +40 days after casing), a decline in laccase levels occurred in the upper-middle layer but it was not until the third harvest (+59 days) that a decline in laccase activity had reached the middle, lower middle and bottom sections. Endocellulase activity was detected at a very high level in the top layer at the onset of the first harvest (Figure 36) simultaneously with laccase activity decline (Figure 37 [cased]). A similar sequence of events was noted in the upper middle layer during the second harvest (+40 days), endocellulase activity rapidly increasing in unison with a rapid decline in laccase activity.

Although, it was the intention to terminate the experiment after three harvests, both columns were kept for a further 16 days before being discarded. During this time, no water had been applied to the casing soil surface of the cased column which allowed the underlying mycelium to develop a surface stroma which became completely impervious to water application. Laccase assays performed on compost extracts taken at this time (+75 days) revealed an increase in enzyme activity in all sections with the uppermost layer returning to a level similar to that recorded prior to mushroom initiation (Figure 37). It would appear from these results, that as the environmental conditions were no longer favourable for fruitbody development, the mycelium had reverted to a vegetative state.

In comparison to the cased column, the uncased column gave high laccase levels in all 5 sections on becoming totally colonised by mycelium (+14 days), although the highest level was detected in the top layer (Figure 37). While there were distinct changes in enzyme activity with depth in the cased column, this was not apparent in the uncased column, where laccase levels generally declined in all 5 sections with time. No endocellulase activity was detected throughout the uncased column during the course of the experiment.

Two bacteriolytic enzymes, β-N-acetyl glucosaminidase and β-N-acetyl muramidase, were assayed in compost extracts taken from the 5 levels of both columns. β-N-acetyl glucosaminidase was generally at its highest level in the uppermost layer of the cased column at the onset of fruiting and for the

first two flushes (Figure 38). In the uncased column activity of this enzyme was generally more uniform with no distinctive peaks in the uppermost layers. β-N-acetyl muramidase showed a higher activity in the cased column (Figure 39) in comparison to the uncased control. It was also interesting to note that on the final sampling date (+75 days), when no fruiting was occurring, the enzyme activity was virtually undetectable as was shown in the uncased column. Although the recorded levels of activity were generally more erratic than was found for β-N-acetyl glucosaminidase, the uppermost layers i.e. top and upper middle, generally gave the highest activities. Both of these enzymes change in activity in unison with endocellulase. This is further proof that the mushroom while totally colonising the column exhausts the substrate of carbohydrate polymers and microbial protein from the uppermost layer downwards. Utilisation of the substrate from the casing soil surface downwards is also reflected by the significant drop in compost moisture in the uppermost layers after 2 flushes, water undoubtedly being required for fruitbody initiation and development (Appendix 14).

Assessment of the debris 1 fractions throughout the course of the experiment are presented in Figure 40 where the mean estimations were determined on 3 replicate samples. Debris 1 estimations, corrected on an constant ash basis, are shown in Figure 41. This gives a clearer picture of the diminishing fractions with time. Debris 1, or the clean straw fraction, declines most rapidly, as would be expected, in the top layer and after two mushroom harvests (+40 days) the upper middle layer shows a major decline. In the uncased control column, the depletion of the debris 1 fraction was much slower and there were very little differences between all 5 sections during the course of the experiment (Figure 41).

Assessment of the debris 2 fractions throughout cropping are presented in Figure 42. As with the analysis of debris 1, mean estimates, once again, were corrected on an equal ash basis (Figure 43). Significant differences in the rate of disappearance of this fraction during cropping with depth were noticeable during the first harvest of mushrooms (+25 days). After the second and third harvests (+40 and 59 days) it was quite clear that this

fraction was least affected at the casing soil surface. The greatest depletion of debris 2 was recorded in the bottom layer of compost where laccase activity was at its highest (Figure 37). Although there was a general fall in the amounts of debris 2 fraction isolated with time in the uncased column, the differences between sample points were less distinct.

Assessment of ash-free humic material for both columns are shown in Figure 44. and the mean estimates once again corrected on an equal ash basis. No major changes with time could be deduced from this set of results. This was also the case for the uncased column, levels of ash-free humic accounting for 6-7% of the total compost dry matter.

5.3 Changes in protein, carbohydrate and phenolic content of ash-free humic in compost samples isolated from cased and uncased compost columns

The levels of (ash free) humic fraction in both cased and uncased columns during mycelial colonisation did not change significantly (Figure 44). This differs from the findings of Wain (1981) who claimed up to 75% of the humic fraction is utilised during the growth and reproductive phases of mushroom production, with the carbohydrate, protein and phenolic components of extracted humic acid being reduced by 94%, 67% and 60% respectively. However, it must be remembered that these estimations were calculated by assuming 16% of the ash free compost dry matter after 26 days of spawn-running was mushroom mycelium dry matter. Adjustments were made by assessing the carbohydrate, protein and phenolic contents of mushroom mycelium and then deducting them from the overall values found in the compost samples.

From race-tube extractions and estimation of laccase activity (see 5.1), mushroom mycelium was estimated to reach levels only equivalent to 7% of the compost dry weight after 41 days. This would be equivalent to approximately 9% of ash-free compost dry matter. Dramatic decreases in carbohydrate, protein and phenolic components of the humic fraction as

calculated by Wain (1981) were not detected, although there was an apparent decline in the protein content of the humic fraction. This amounted to a percentage reduction of 20% (i.e. a fall from 50% to 40%) and occurred between day 25 and day 40 in compost samples isolated from the cased column (Figure 45), a time when fruitbody production was at its highest. In comparison to this, the protein content of humic material isolated from the uncased column also declined, but to a lesser extent. Analysis of the carbohydrate component of humic material isolated revealed only minor changes in the uncased compost samples throughout the growth phase whereas the results for the cased column were more variable (Figure 46). Variations in the carbohydrate content of the humic fraction of compost samples taken from the cased column could possibly be explained by the fact that more critical changes were occurring in this column i.e. fruitbody production, in comparison to the vegetative growth maintained in the uncased column. As mushroom mycelium sequentially degrades its substrate with extracellular enzyme activity, it is possible that nutrients necessary for fruitbody development (short chain carbohydrates) were released into the compost. Increases in the carbohydrate component of the humic fraction were detected in all layers of the cased column, especially in the latter stages of mushroom production (Figure 46). In the uncased column the carbohydrate level of the humic fraction, in all three layers sampled, remained at a level close to that found in the colonised compost at the start of the experiment.

There was also evidence that the phenolic content of the humic complex slowly decreased during the mycelial growth phase in compost samples taken from the cased layer (Figure 47) and to a lesser extent in the uncased layer.

5.4 Concluding Comments

In the previous chapter, studies were concentrated on the changes in humic, fulvic, debris 1 and debris 2 fractions accumulating during composting. It became quite clear that humic and more significantly the debris 2 fraction

Figure 36. Extracellular endocellulase activity within 5 layers of the cased compost column

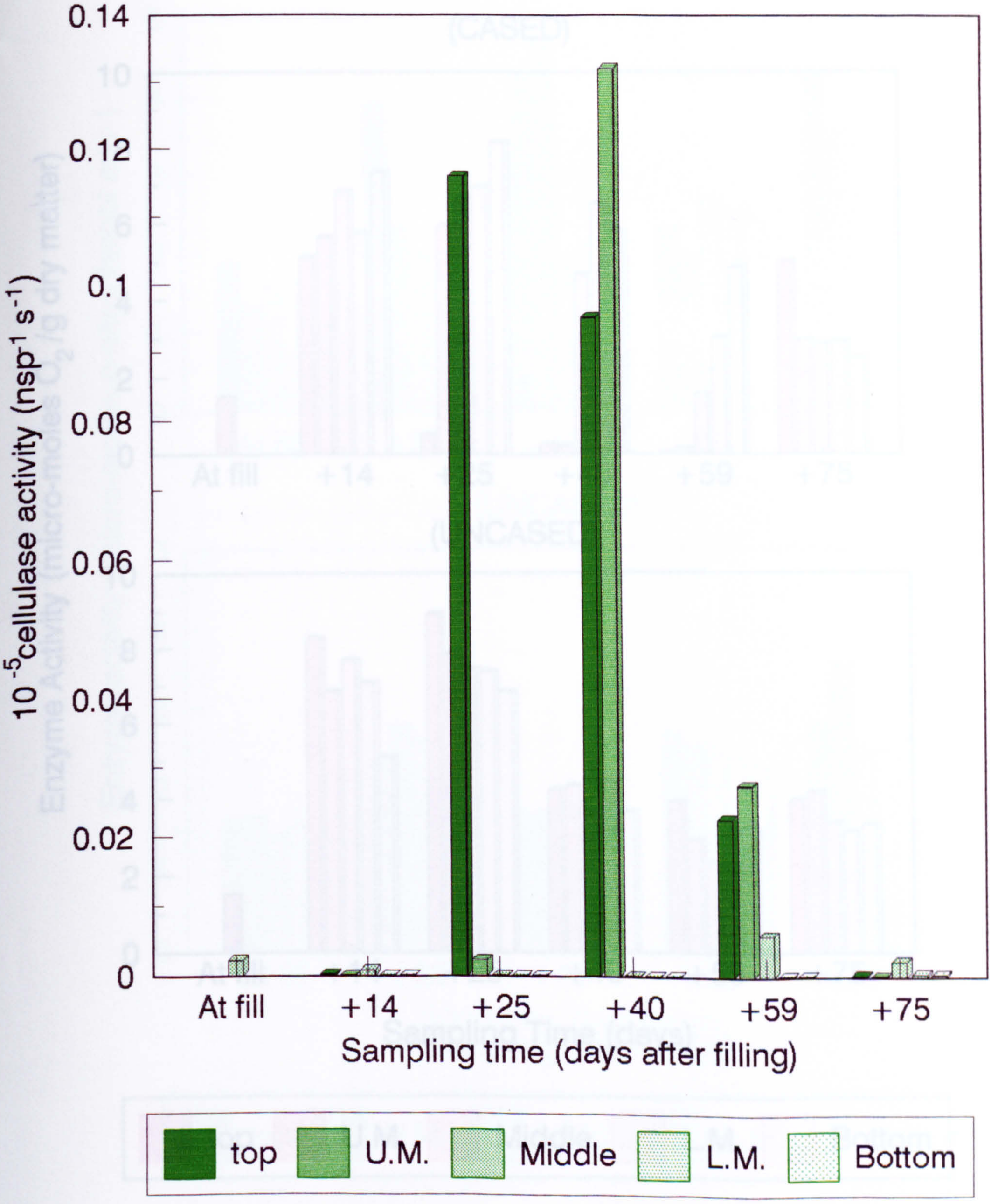


Figure 37. Laccase assays on cased and uncased compost columns

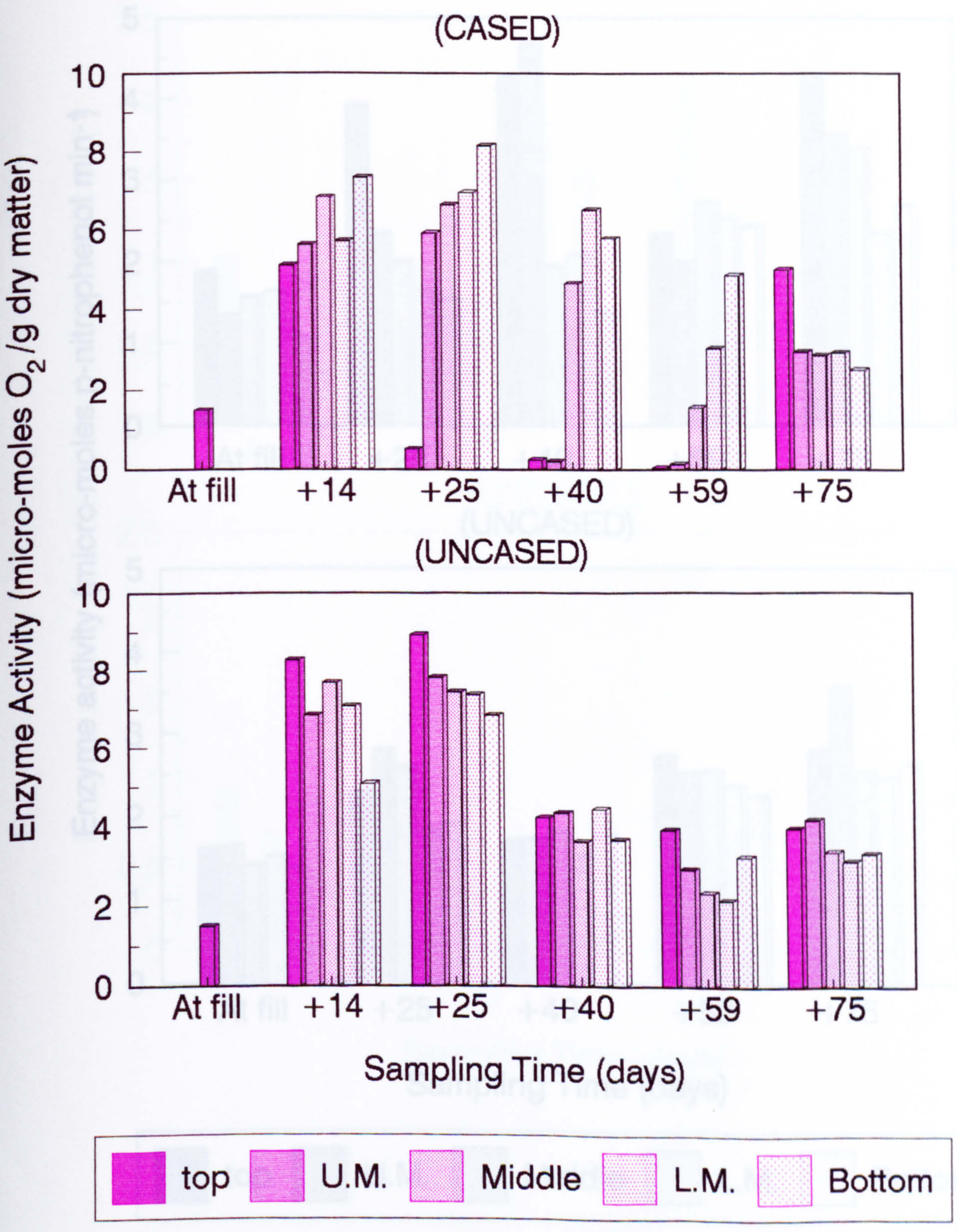


Figure 38. Acetyl glucosaminidase activity in cased and uncased compost columns

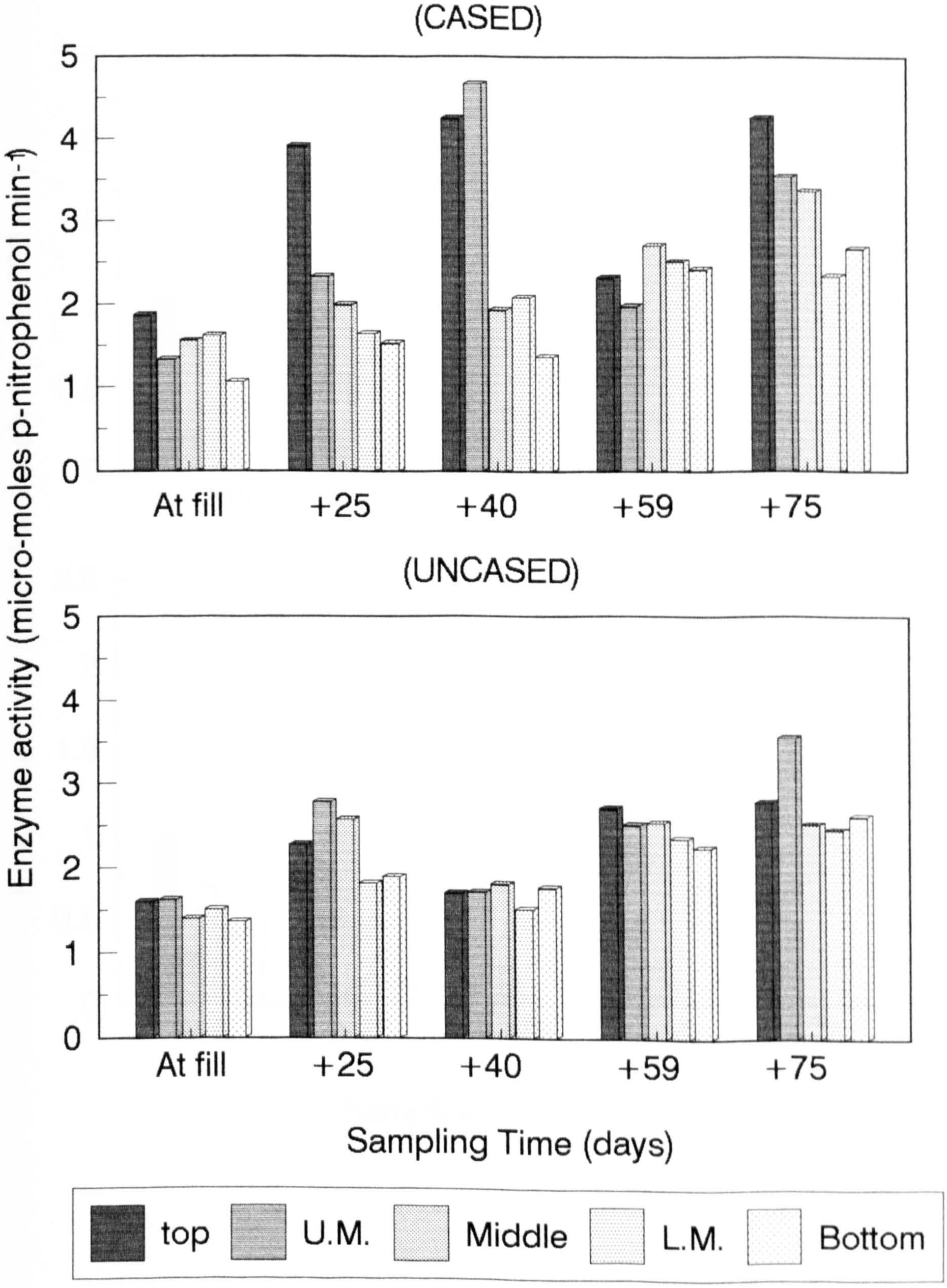


Figure 39. Acetyl muramidase activity in cased and uncased compost columns

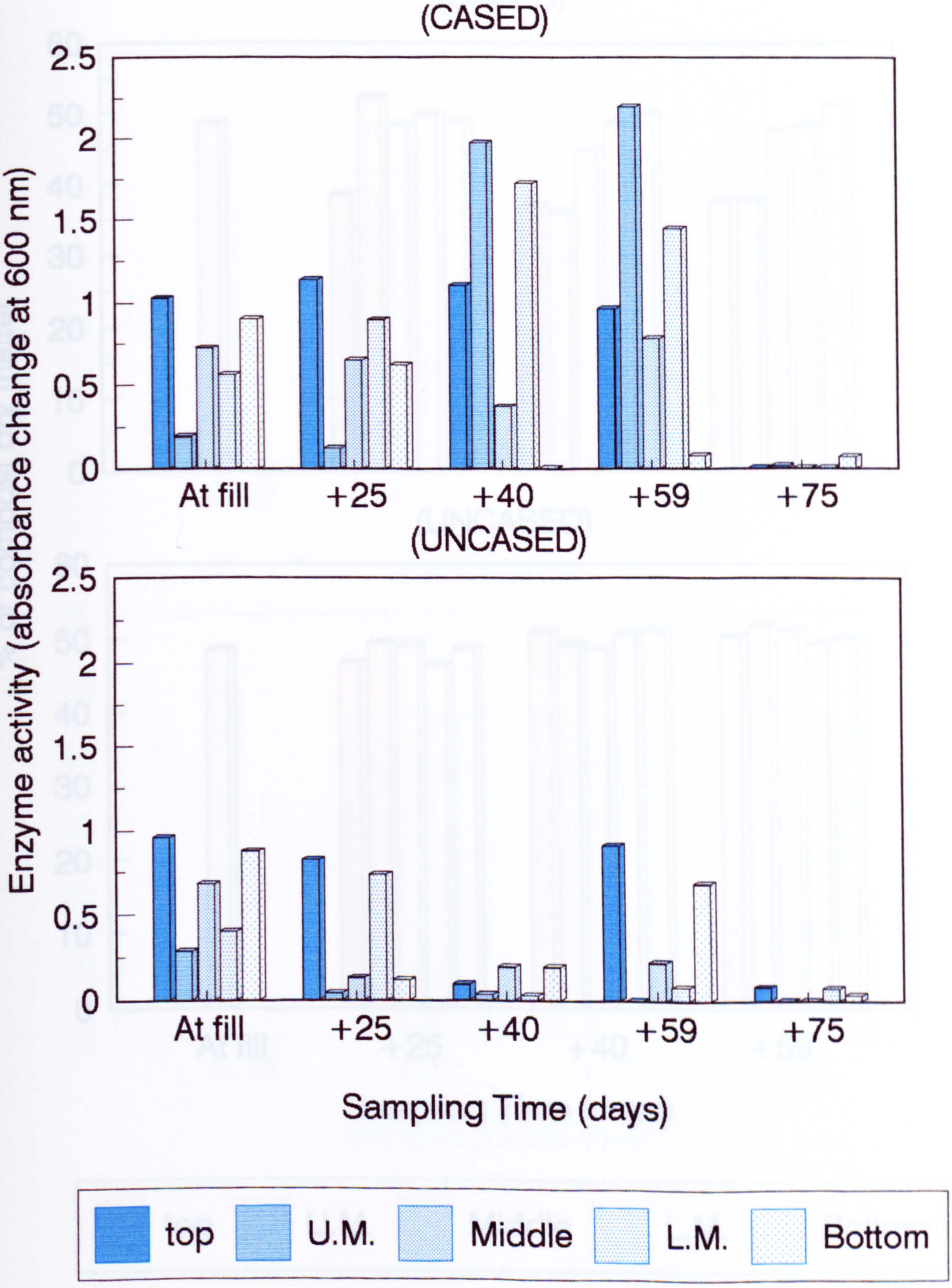


Figure 40. Utilisation of Debris 1 by mushroom mycelium

within 5 separate compost layers of the deep column

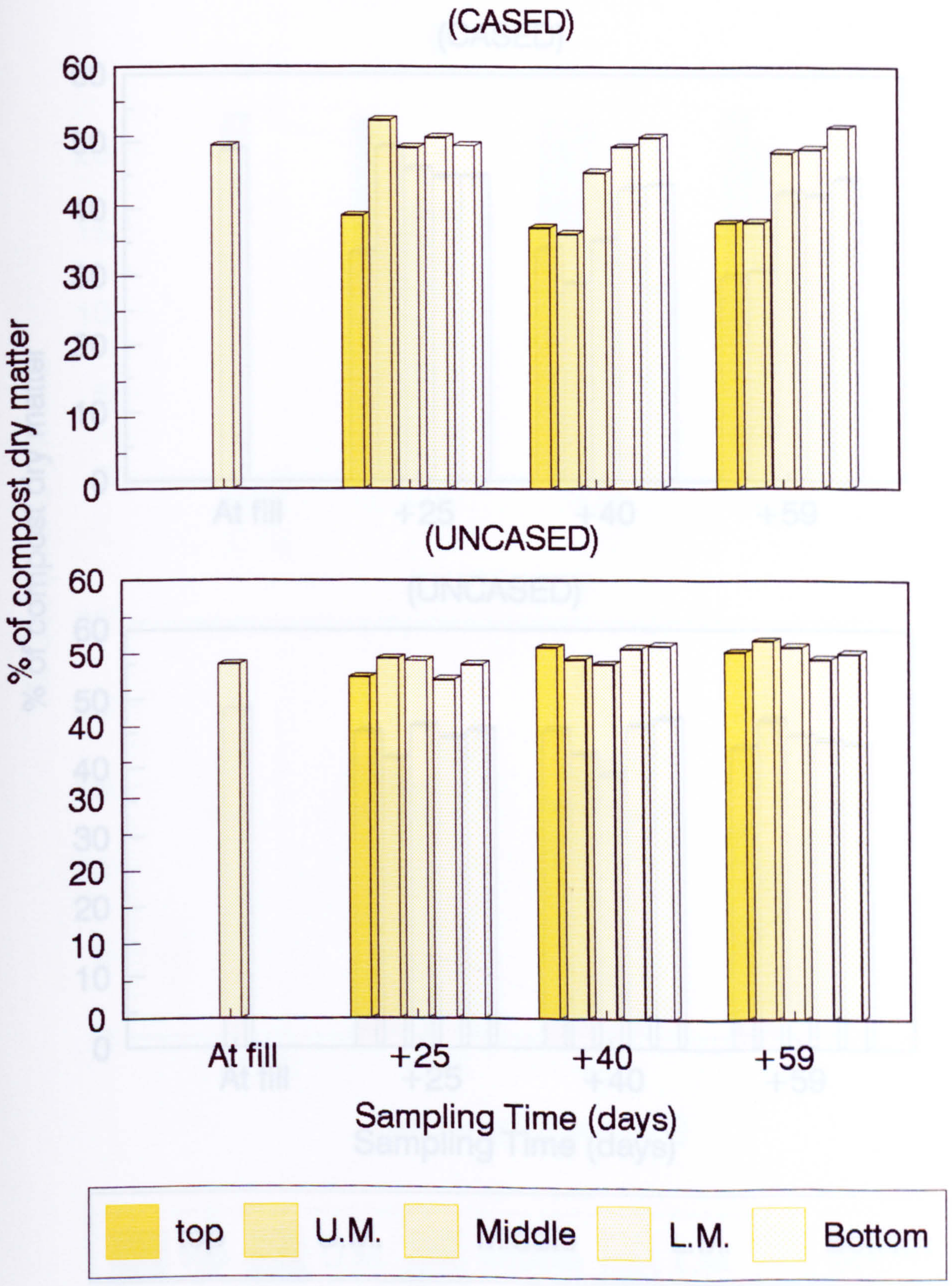


Figure 41. Utilisation of Debris 1 by mushroom mycelium

within 5 separate compost layers of the deep column

(corrected for constant ash)

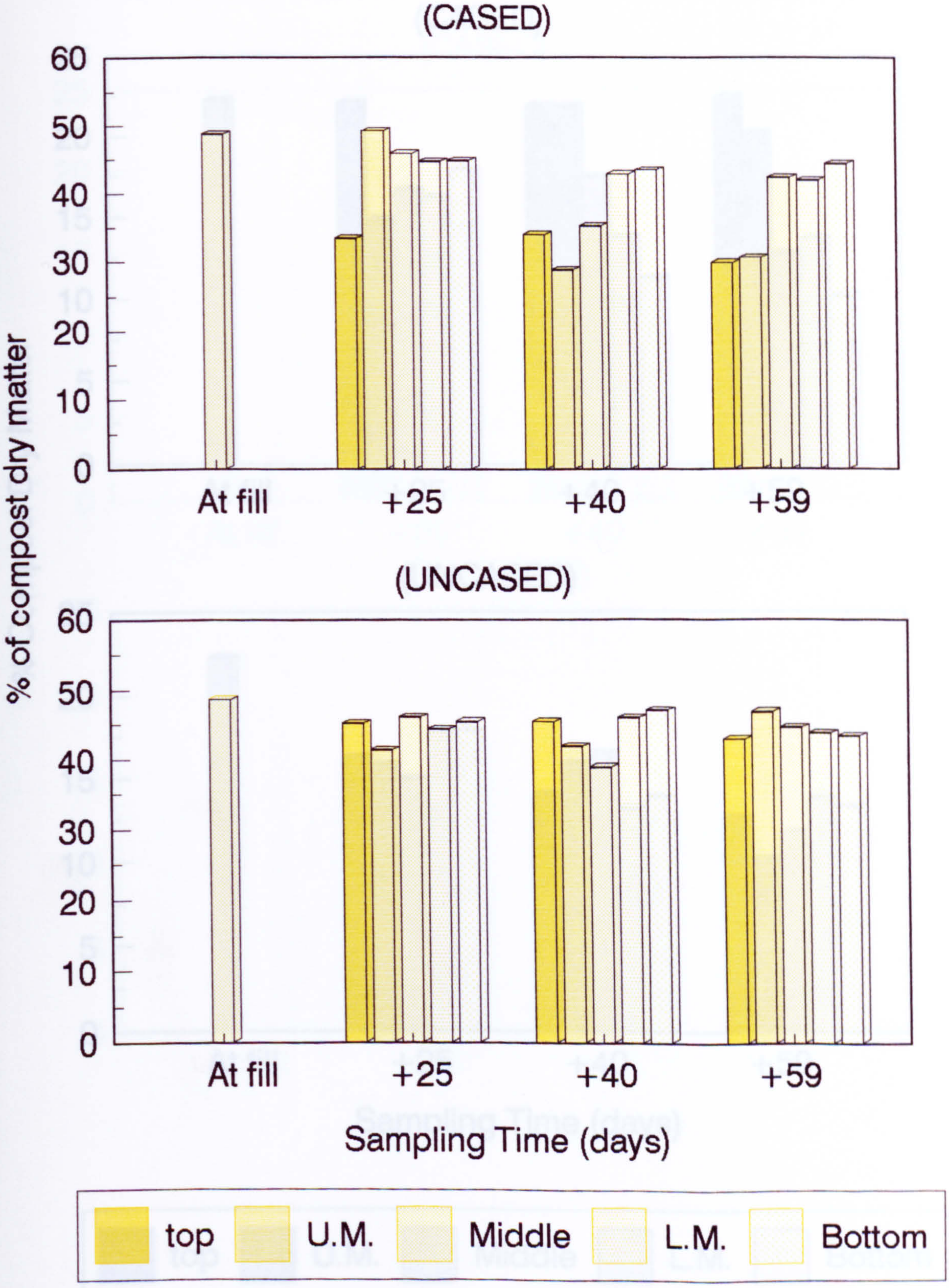


Figure 42. Assessment of Debris 2 remaining within
5 separate compost layers of the deep column

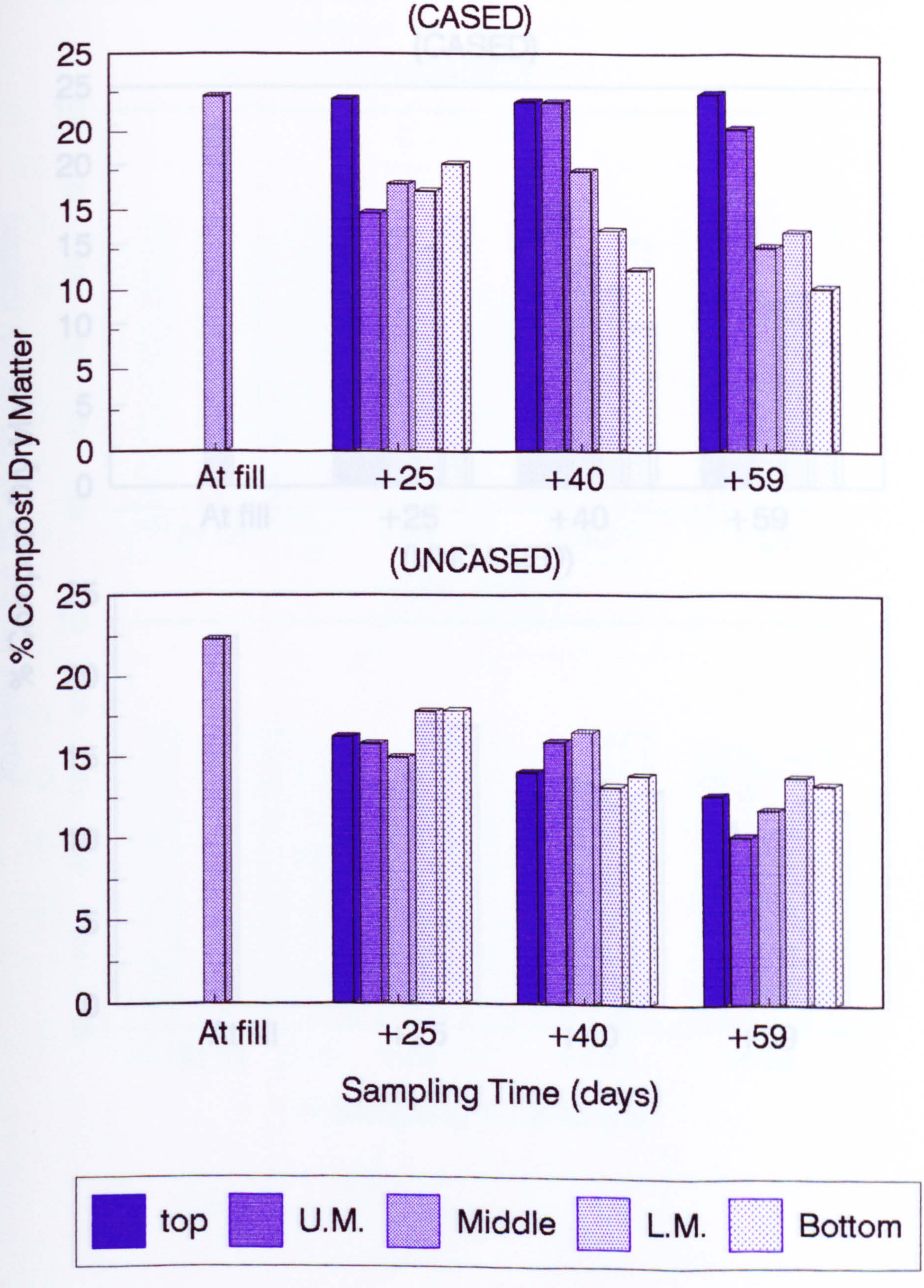


Figure 43. Assessment of Debris 2 remaining within 5 separate compost layers of the deep column

(corrected for constant ash)

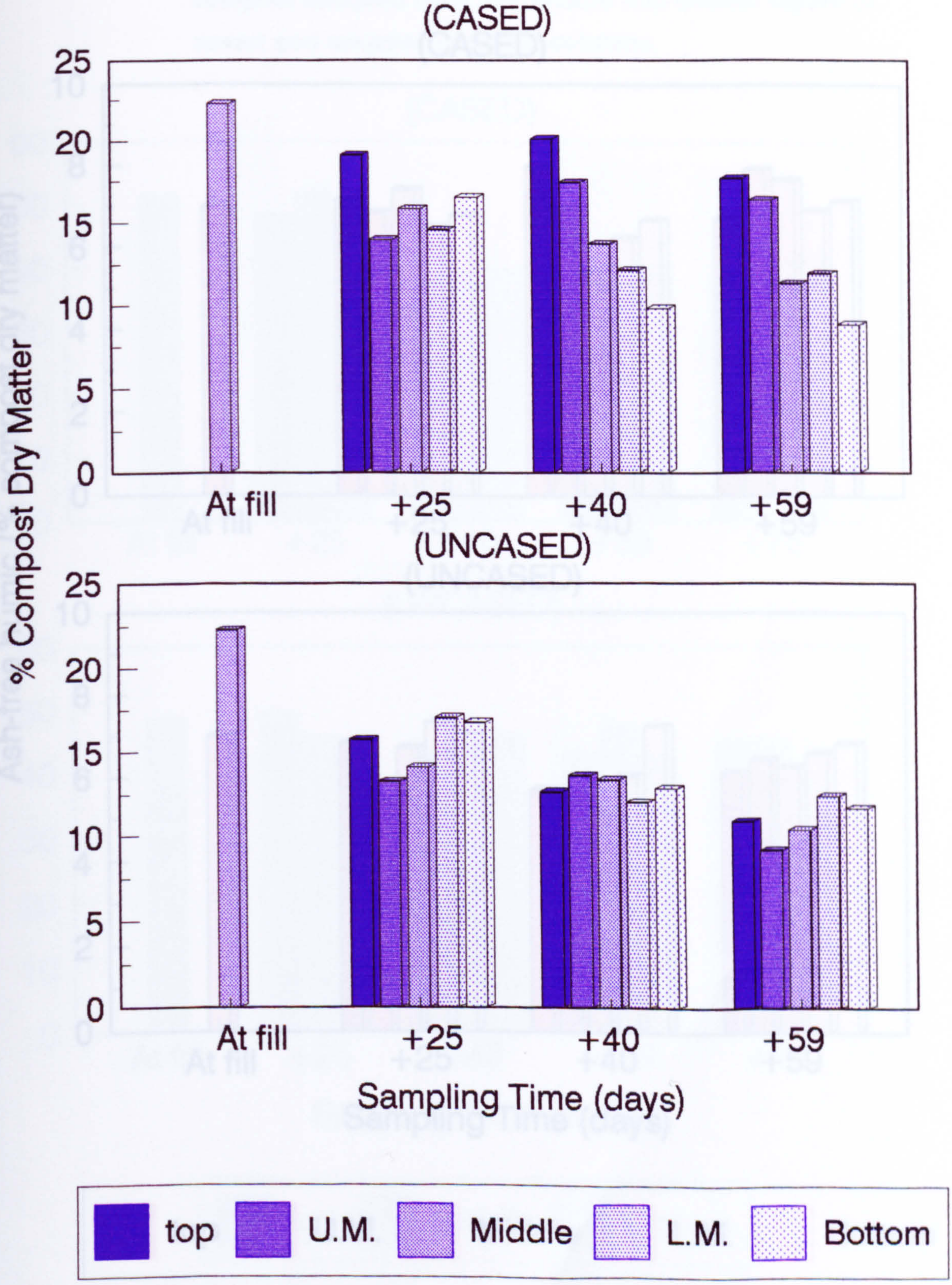


Figure 44. Assessment of Ash-Free Humic material within 5 separate compost layers of the deep column

(corrected for constant ash)

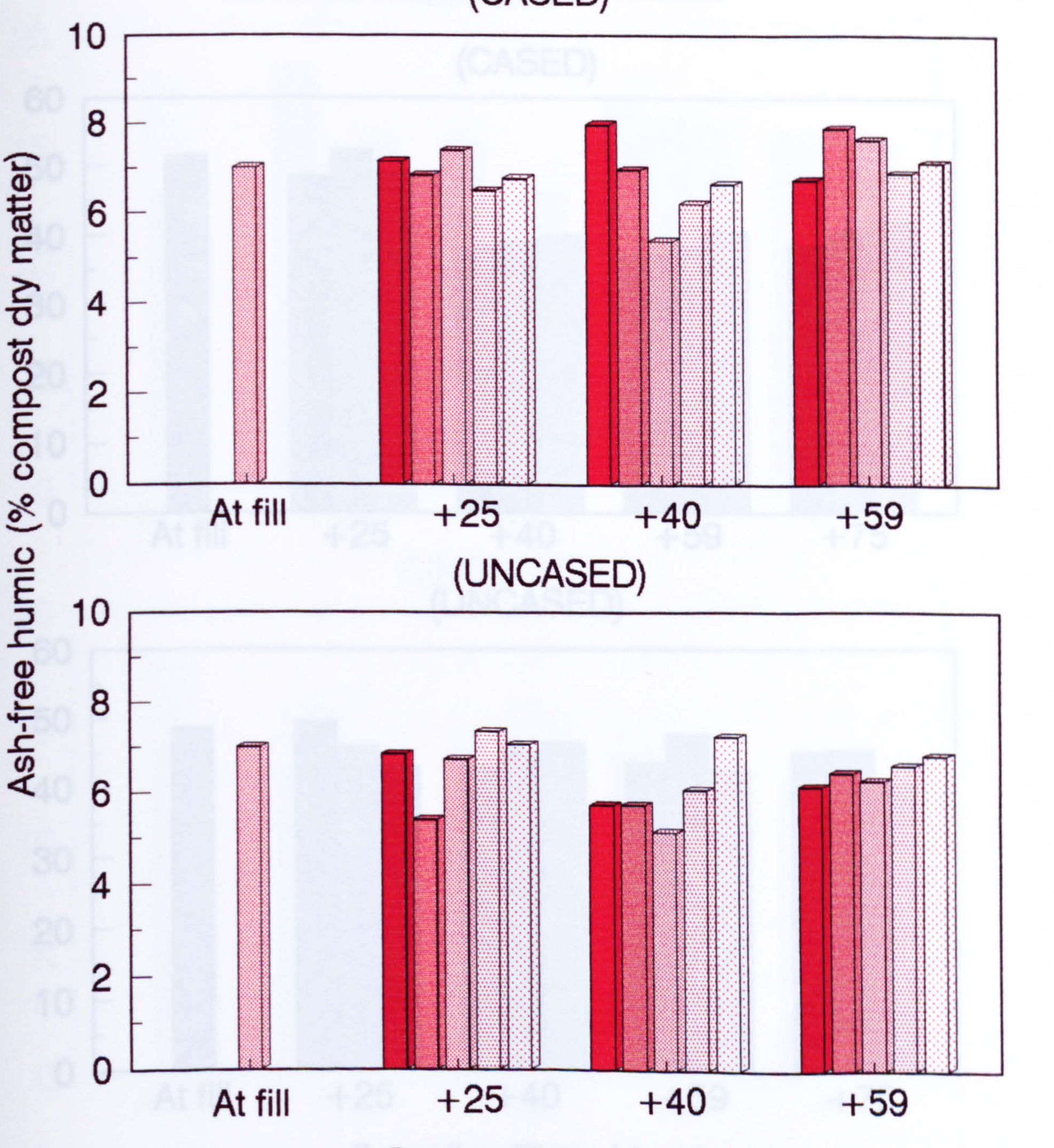


Figure 45. Protein content of Ash-Free Humic material of compost sampled from top, middle and bottom layers of cased and uncased compost columns

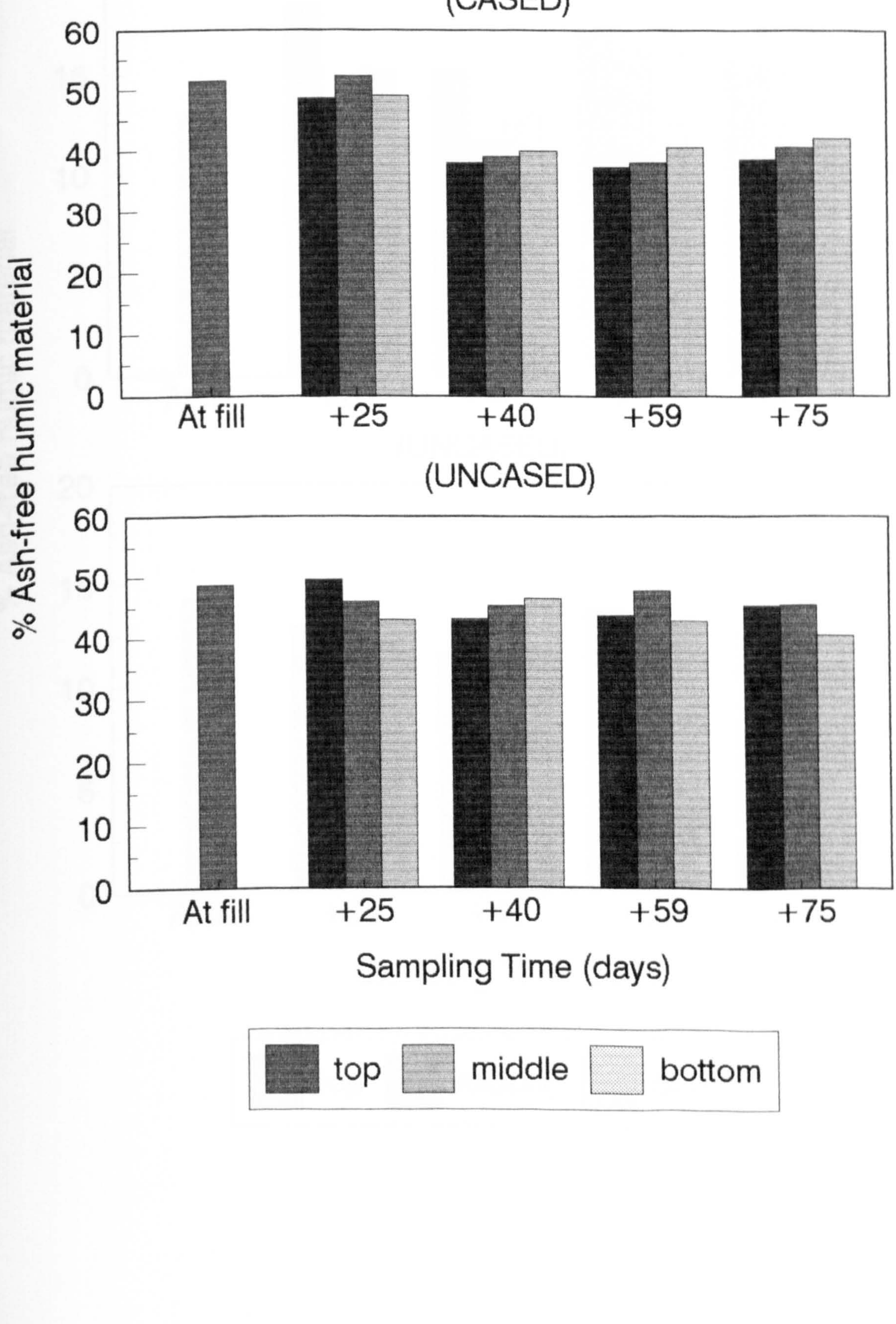


Figure 46. Carbohydrate content of Ash-Free Humic material of compost sampled from top, middle and bottom layers of cased and uncased compost columns

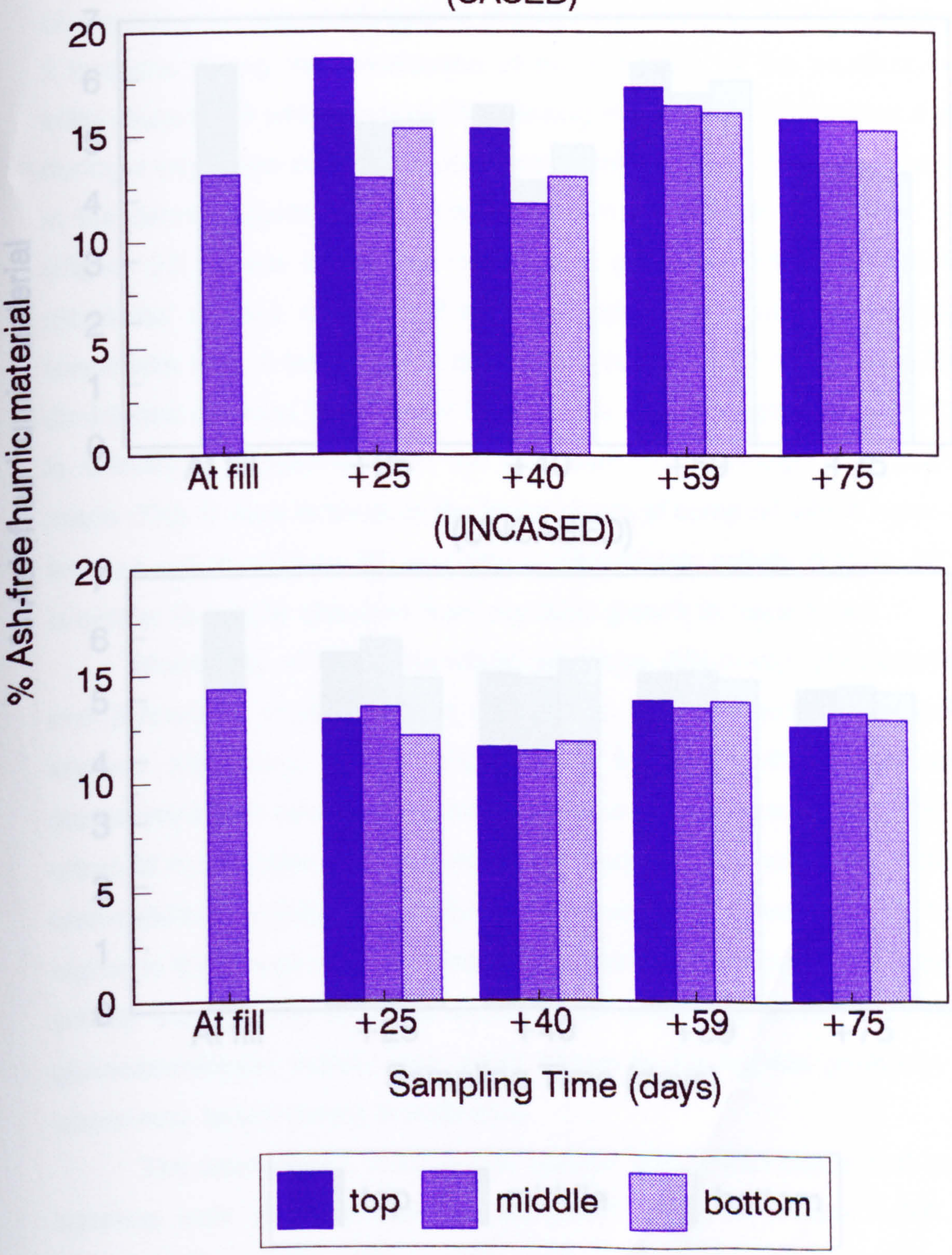
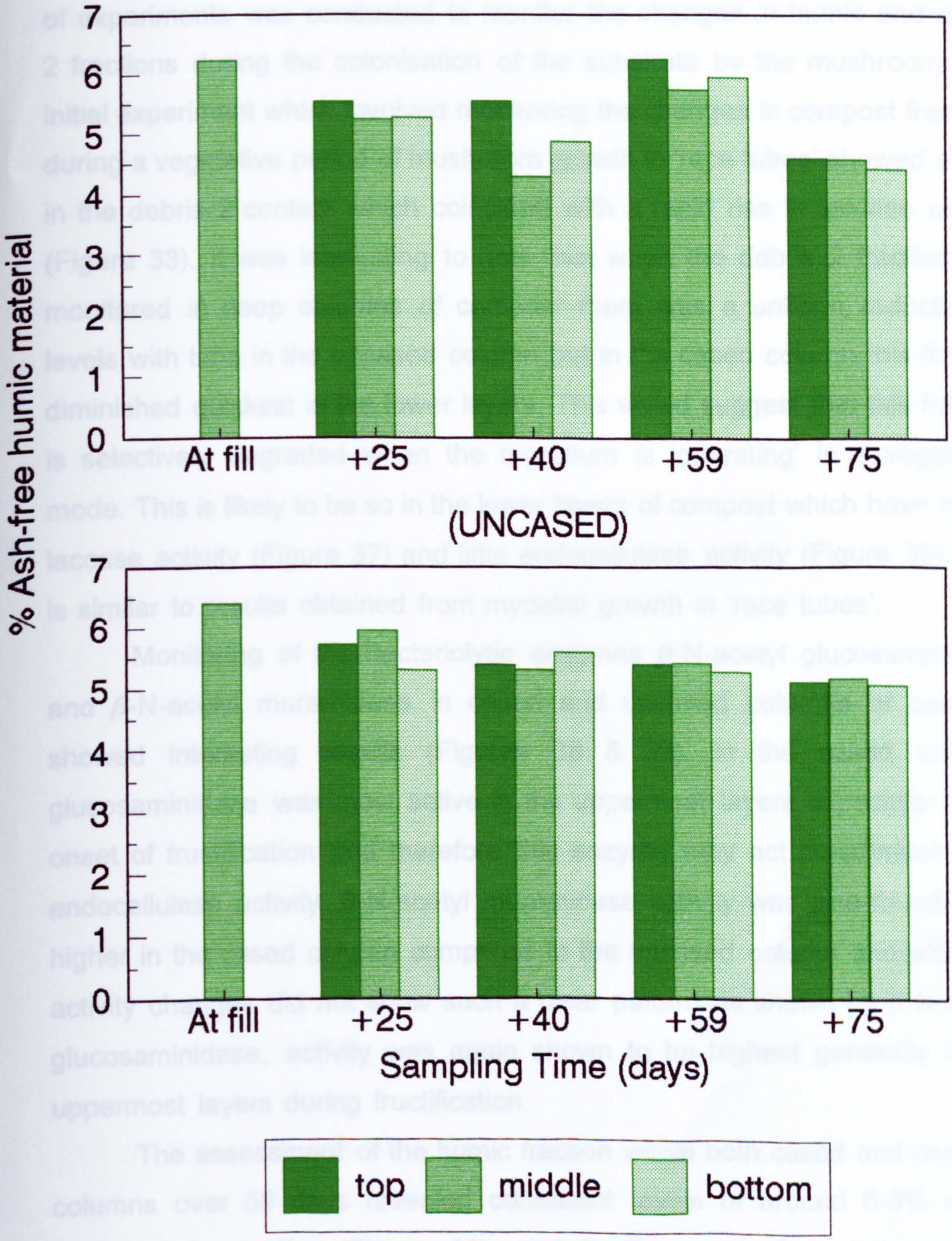


Figure 47. Phenolic content of Ash-Free Humic material of compost sampled from top, middle and bottom layers of cased and uncased compost columns



increased markedly so that they represented approximately 10% and 30% of the compost dry matter respectively at the time of inoculation. It was also suggested that these fractions were probably important to the nutrition of the mushroom as at the time of spawning the nitrogen content of these two fractions amounted to 40% of the total. To substantiate these claims, a series of experiments was conducted to monitor the changes in humic and debris 2 fractions during the colonisation of the substrate by the mushroom. The initial experiment which involved monitoring the changes in compost fractions during a vegetative period of mushroom growth in 'race tubes' showed a drop in the debris 2 content which coincided with a rapid rise in laccase activity (Figure 33). It was interesting to note that when the debris 2 fraction was monitored in deep columns of compost there was a uniform reduction in levels with time in the uncased column but in the cased column this fraction diminished quickest in the lower layers. This would suggest that this fraction is selectively degraded when the mycelium is 'operating' in a vegetative mode. This is likely to be so in the lower layers of compost which have a high laccase activity (Figure 37) and little endocellulase activity (Figure 36). This is similar to results obtained from mycelial growth in 'race tubes'.

Monitoring of the bacteriolytic enzymes β-N-acetyl glucosaminidase and β-N-acetyl muramidase in cased and uncased columns of compost showed interesting results (Figures 38 & 39). In the cased column, glucosaminidase was most active in the uppermost layers especially at the onset of fructification and therefore this enzyme may act coordinately with endocellulase activity. β-N-acetyl muramidase activity was also found to be higher in the cased column compared to the uncased column and although activity changes did not show such a clear pattern as shown by β-N-acetyl glucosaminidase, activity was again shown to be highest generally in the uppermost layers during fructification.

The assessment of the humic fraction within both cased and uncased columns over 59 days revealed consistent levels of around 6-8% of the compost dry matter (Figure 44), and there appeared to be no major reductions in ash-free humic material during colonisation or fruitbody

production. However it was shown that humic material extracted by sonication and precipitation at pH 2 consisted of approximately 50% protein, and that the protein content of the humic fraction did show a marked drop of around 20% after two flushes had been harvested in all three layers sampled (Day 40; Figure 45). Reductions in the protein content of the humic fraction isolated from uncased compost samples were also found, but these were less distinct.

CHAPTER 6 GROWTH OF MUSHROOM MYCELIUM ON SOLID AND LIQUID MEDIA

6.1 Mycelial growth on water agar supplemented with humic and debris 2 fractions as the sole nutrient source.

In order to obtain sufficient levels of both humic and debris 2 fractions to perform nutrient tests on agar, compost samples (0.5 kg fresh weight) were extracted by shaking gently with 500 ml volumes of 0.5 M NaOH (See 2.9.1). Generally the debris 2 (Plate 14) and humic fractions were isolated in the ratio of approximately 0.85:1 respectively, although it should be noted that a somewhat higher ratio (1.5:1) was found when freeze dried compost samples were sonicated in 0.5M NaOH for 10 minutes (see Control composts, Appendix 2 & 3).

To test whether humic and debris 2 fractions were major contributors to the growth of mushroom mycelium, both fractions were isolated and added independently (1g/25 ml) or combined (0.5g each/25ml) to water agar. Potassium dihydrogen phosphate (KH_2PO4;136mg/100 ml) and disodium hydrogen phosphate (Na_2HPO4;142mg/100 ml) were also added to the agar media to ensure a pH close to neutrality. The mycelial radial growth from 3mm diameter inoculum plugs (Horst U3) on all three treatments was compared with mycelial growth on 2% malt agar plates (10 replicate plates per treatment). A further control treatment i.e. water agar alone, was used to assess mycelial growth away from a high nutrient source (the inoculum plug itself) onto water agar completely devoid of nutrients.

It was quite clear that both the humic and debris 2 fractions alone would support mycelial growth, but not to the extent of the malt control medium (Figure 48). It was also noted that while debris 2 gave a significantly better growth than the humic fraction, when both were added to water agar at a 1:1 ratio, there was a further synergistic improvement which was significant in this experiment at the 1% level.

6.2 Mycelial growth on minimal glucose media supplemented with either humic or debris 2 fractions as the sole nitrogen source.

As both humic and debris 2 fractions are rich in nitrogen (ca. 7.5% and 3% respectively - see Tables 16 & 18), increasing levels of these fractions, alone and in combination were added to buffered 1.5% glucose agar, as the sole nitrogen source, and their effect on radial growth was measured. As with the previous experiment, a control (2% malt agar) was included as a comparison. Mycelial growth rate on the 1.5% glucose agar control, was not surprisingly, poor, (less than 1 mm/day), due to nitrogen deficiency. There were significant improvements in growth rate when humic material was added to the minimal medium, although there was no apparent improvement in growth when the supplementation rate was increased from 0.125g to 0.5g/25 ml agar media (Figure 49). Supplementation of the glucose minimal media with debris 2 also improved growth rate (Figure 50) with the highest supplementation rate giving the largest increase. When both fractions were combined in a 1:1 ratio (0.25g of each/25 ml agar), growth rate improved significantly with increasing supplementation level (Figure 51) but it would appear from the combined findings that the debris 2 fraction was a better source of nitrogen than the humic fraction. Growth rate once again in comparison to the 2% malt control series was still relatively poor.

6.3 Induction of proteinase by mushroom mycelium when grown in defined liquid media (Treschow's) using humic fraction as the sole nitrogen source.

To demonstrate that the humic fraction could be an important source of nitrogen for *Agaricus bisporus*, extracted humic material was added to a medium based on Treschow (1944) as a nitrogen replacement for glutamate. Sufficient media was prepared to fill 35 x 100 ml conical flasks. Two further series of flasks (35 reps) containing (a) the standard Treschow's medium and (b) casein - supplemented Treschow's medium lacking glutamate, were also

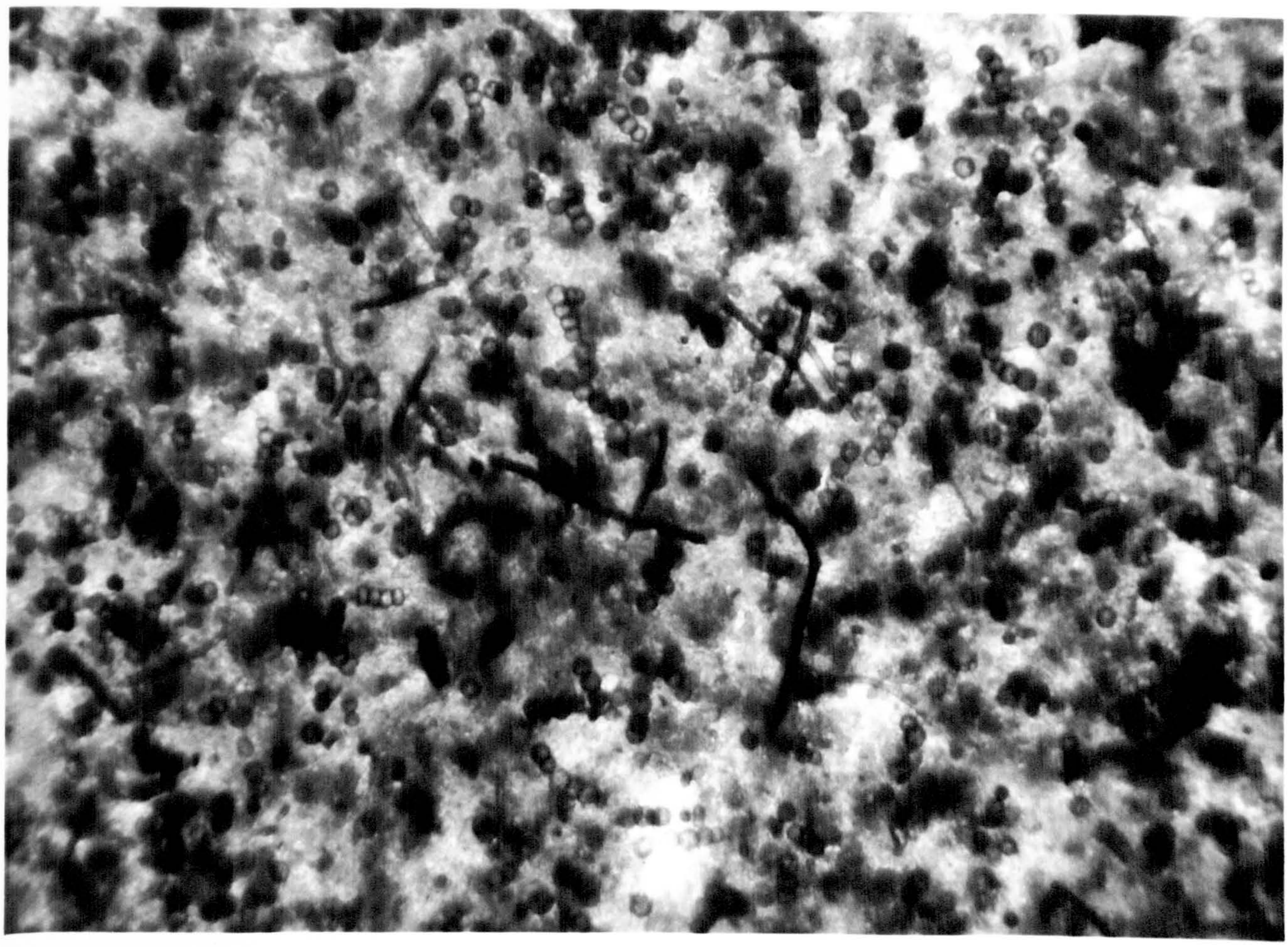


Plate 14. Debris 2 fraction isolated from fresh weight compost sample (x 200). N.b. high proportion of bacterial cells and fungal fragments.

Figure 48. Growth of Mushroom Mycelium on Water Agar

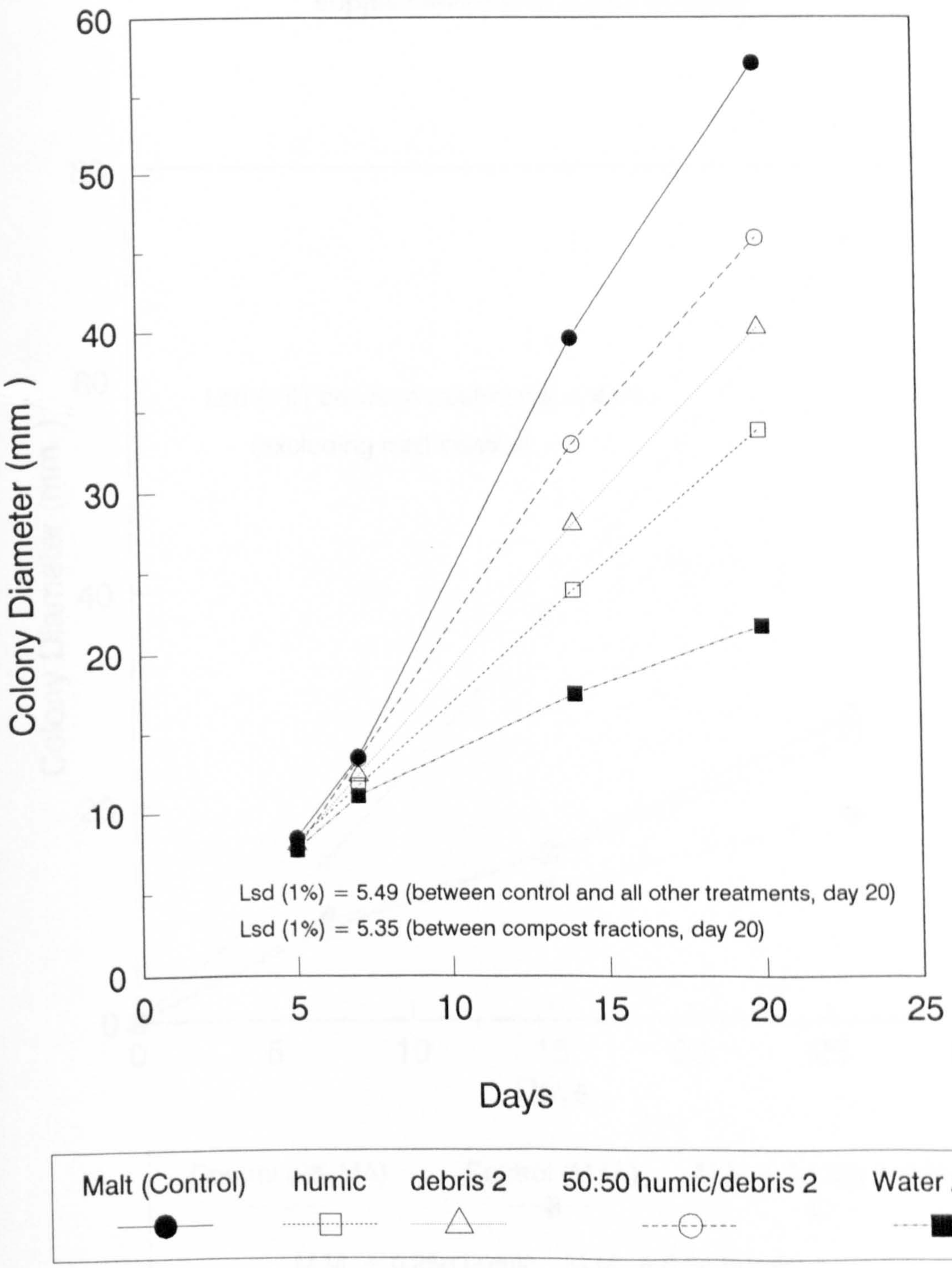


Figure 49. Growth of Mushroom Mycelium on Minimal Medium

supplemented with humic material

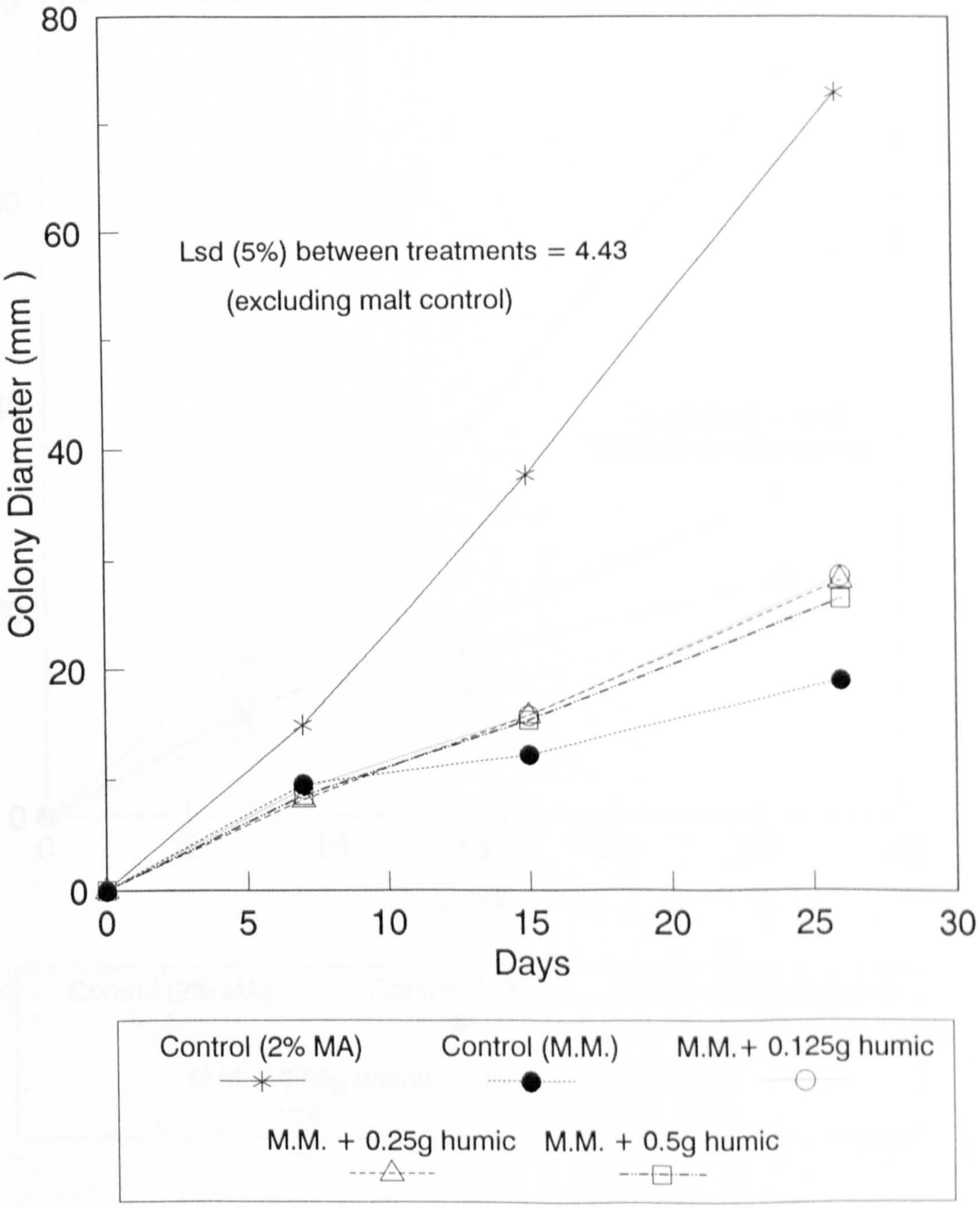


Figure 50. Growth of Mushroom Mycelium on Minimal Medium

supplemented with debris 2

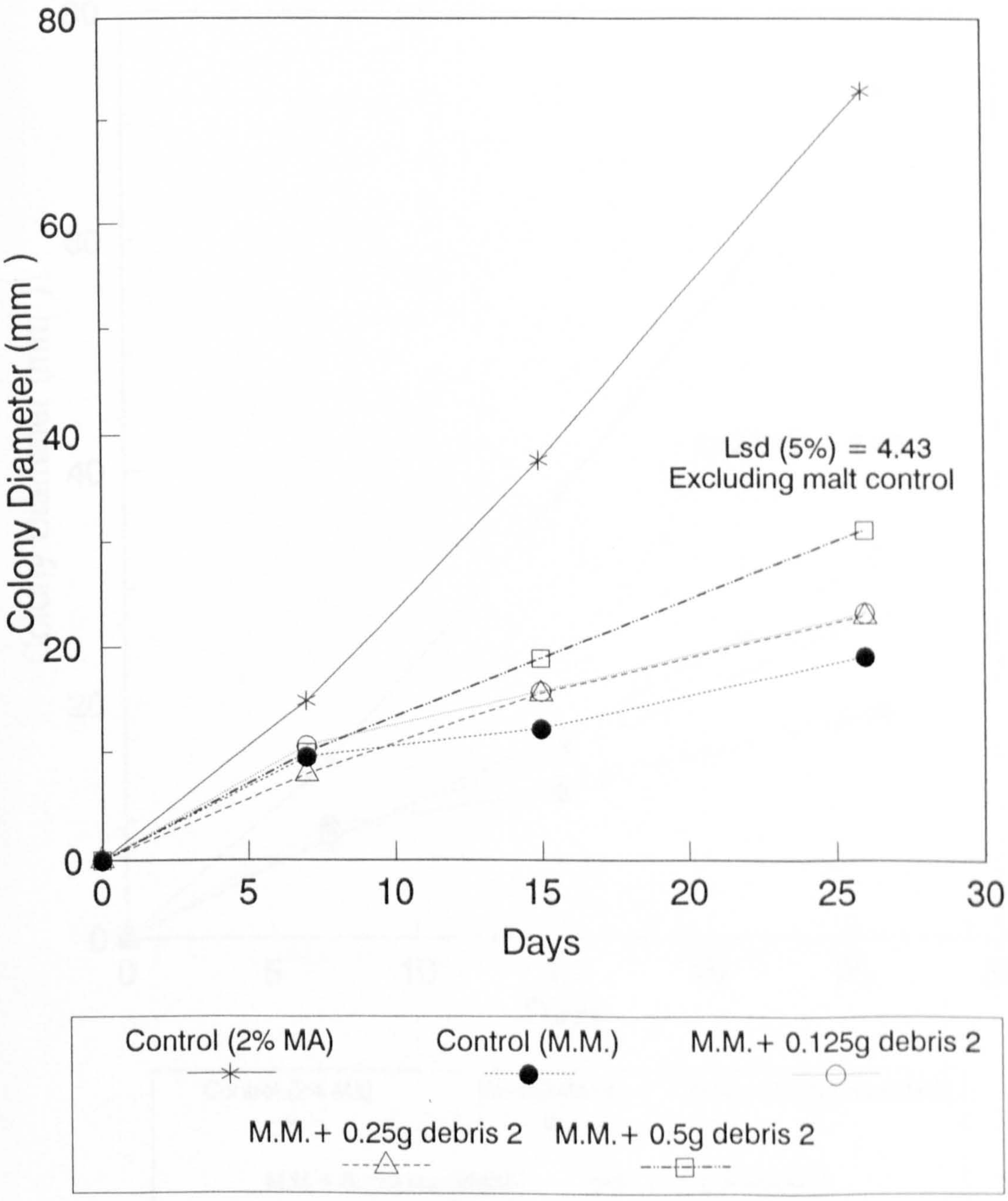


Figure 51. Growth of Mushroom Mycelium on Minimal Medium

supplemented with humic/debris 2 mixtures

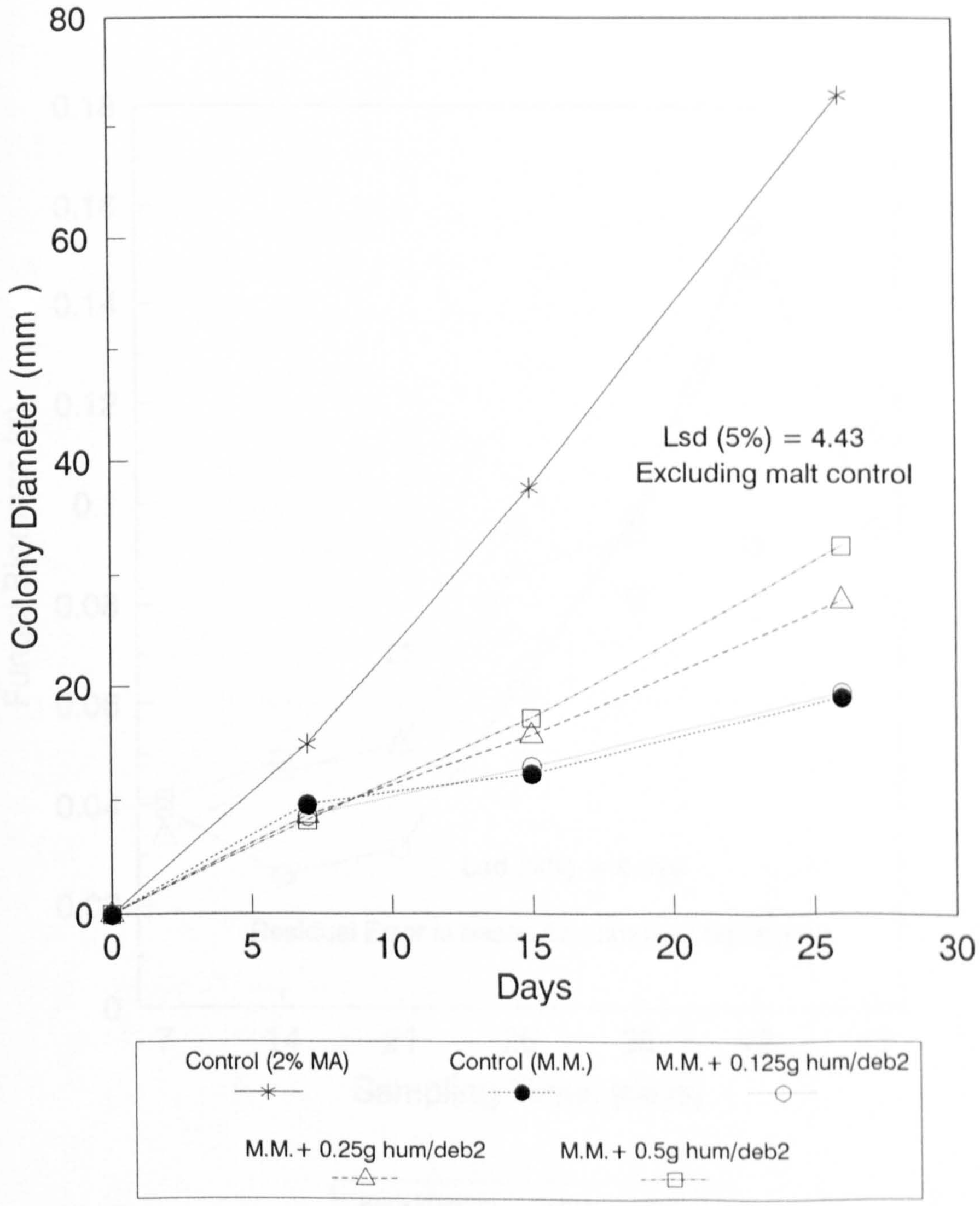


Figure 52. Fungal Biomass accumulation in Treschow's Medium when Glutamate (sole N source) is replaced by Humic Acid and Casein

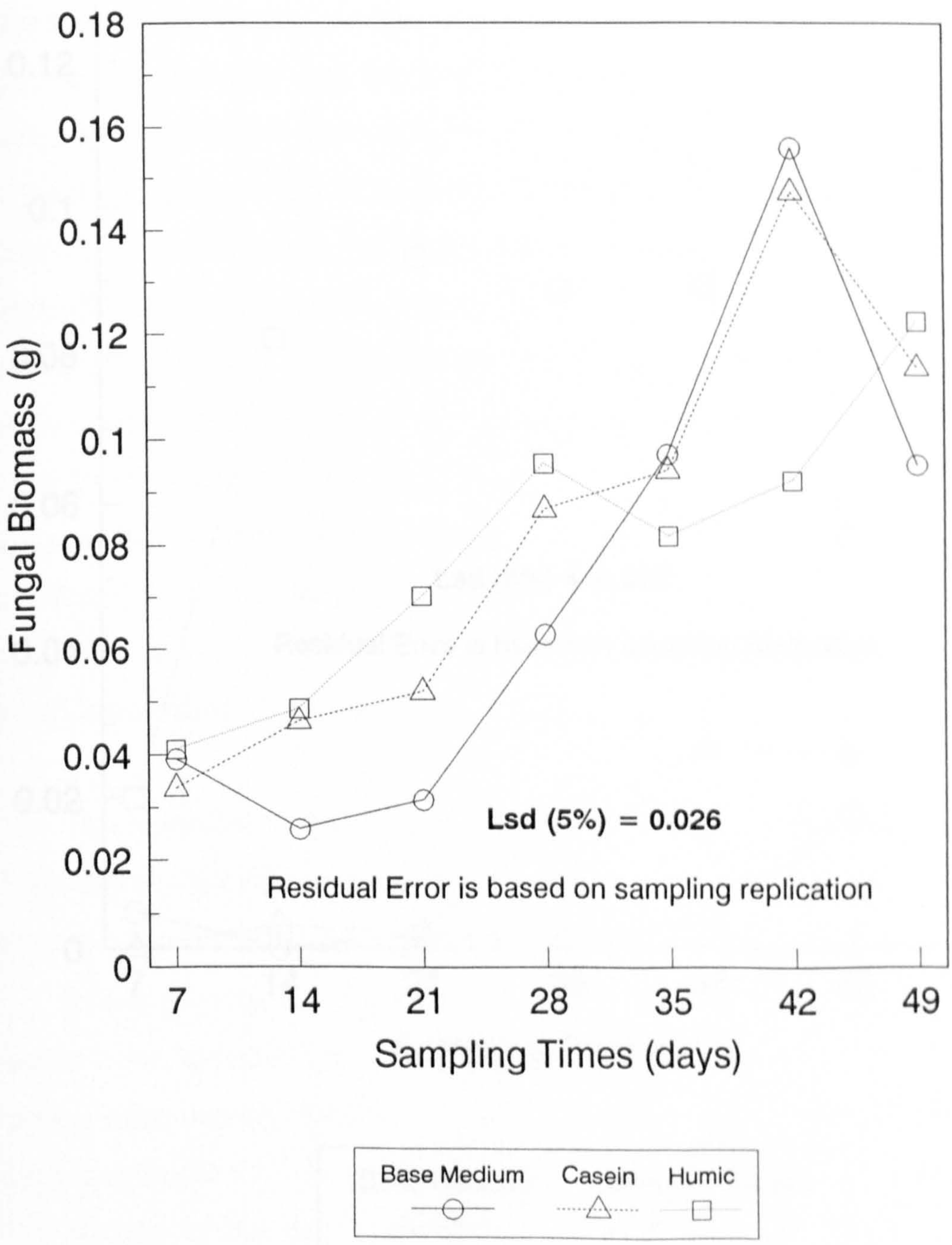
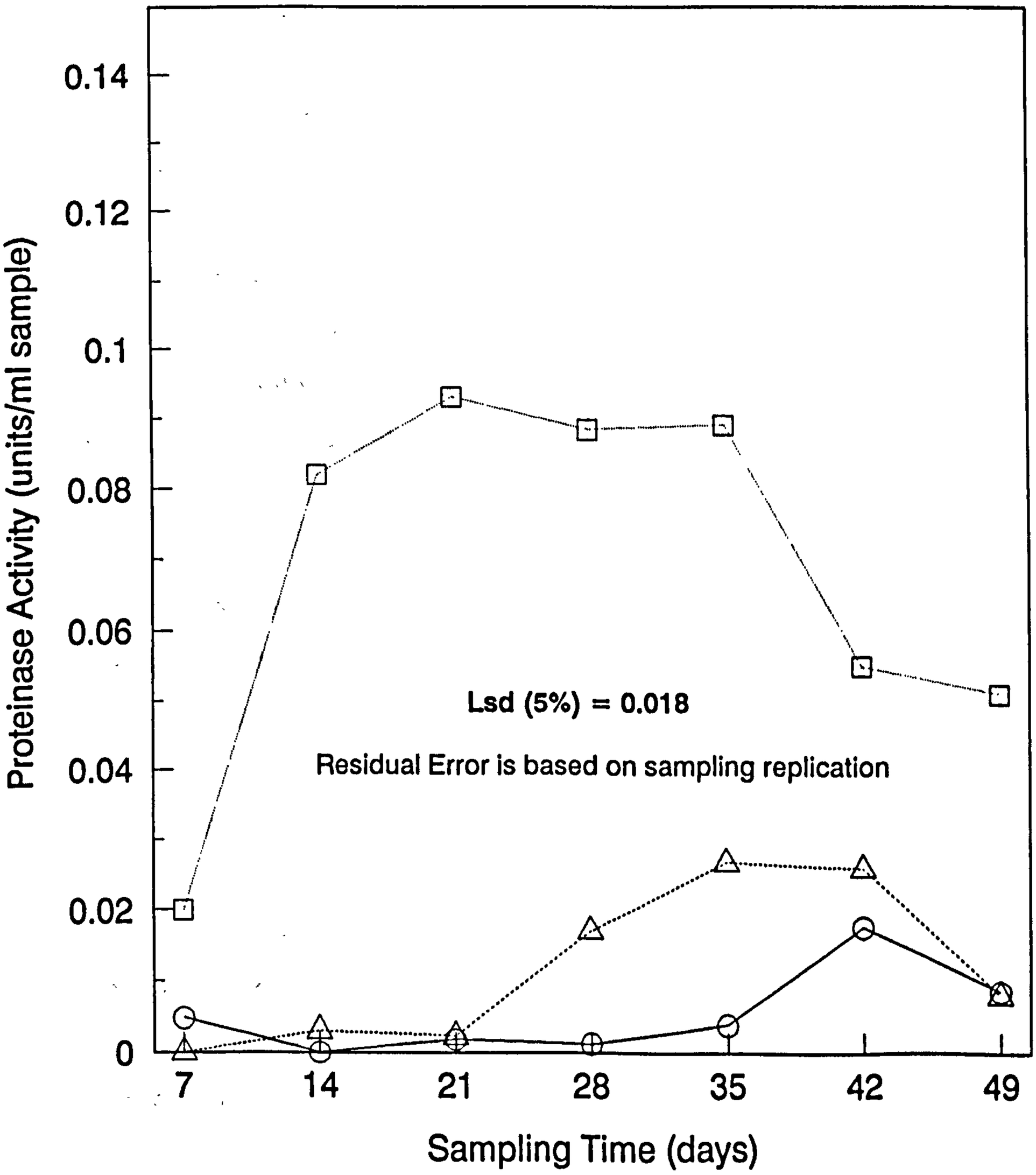


Figure 53. Induction of Proteinase in Treschow's Medium

when glutamate (sole N source) is replaced by humic acid and casein



prepared (See Appendix 1). Two mycelial plugs (5mm diameter) of *A.bisporus* were added to each flask and all flasks were incubated at 25°C. Five flasks were randomly selected at 7 sampling times i.e. after 7, 14, 21, 28, 35, 42 and 49 days and the mycelial contents filtered. The clear supernatants were used to assay proteinase activity (see 2.15).

Mycelial growth in the standard Treschow medium was visibly slower in the first 14 days when compared to the media supplemented with humic material and casein and this was confirmed when mycelial dry weights were determined (Figure 52). Proteolytic activity was assayed spectrophotometrically on the supernatants after 7 days when no activity could be found on the standard Treschow medium containing glutamate or the media supplemented with casein, but there was some evidence of activity in the medium containing humic material. This was confirmed after 14 days (Figure 53) when proteolytic activity was clearly evident and this increased even further after 21 days. Although the humic fraction had little effect on growth under these conditions, it was clearly a stronger inducer of proteinase activity than casein. Proteinase activity in the casein supplemented medium was detected first after 28 days and this corresponded with clearing zones in precipitated casein at the base of the flasks.

6.4 Concluding Comments

The objective of the solid and liquid media experiments reported in this section were to provide additional, supportive evidence, that humic and debris 2 fractions were important nutrient sources for mushroom nutrition.

In experiment 6.1, both the humic and debris 2 fractions were added to water agar, independently and together, as the sole nutrient source. Both fractions were shown to support mycelial growth, but the debris 2 fraction was shown to support a significantly quicker growth than the humic fraction. When a 1:1 mixture of the two fractions were added to water agar, there was a significant synergistic effect recorded but the radial growth measurements were still below that of the 2% malt control.

In experiment 6.2, humic and debris 2 fractions were added independently and together as the sole N sources at increasing levels to a minimal glucose medium. As this medium was enriched with a carbon source, supplementation rates for debris 2 and the humic fraction were not as high as that used in experiment 6.1. This may have contributed to the slower growth rates, but the overall conclusion, confirming the observations of experiment 6.1, was that the addition of either fractions to the base media improved mycelial growth rate and a 1:1 mixture of the two gave marginally the highest growth rate.

As the humic fractions precipitated from alkaline extracts were shown to contain upwards of 40% protein, this material was included in a defined liquid medium as the sole nitrogen source and a replacement for glutamate (experiment 6.3). A second treatment using casein was also prepared as a further control. All three media were assayed for proteinase over a 7 week period. Proteinase activity was detected after only 7 days in the humic supplemented medium reaching its highest level after 21 days whereas activity in the casein supplemented medium was only beginning to be detected at this stage. The most important conclusions that can be made from this experiment, are that the humic complex is a good inducer of proteinase and it is highly likely that the nitrogen within this complex contributes to nutrition of the mushroom.

CHAPTER 7

DISCUSSION

7.1 Mycelial growth in compost and how it relates to compost selectivity

Although a great deal is now known regarding the microbial groups dominating mushroom composts and how they utilise the readily available carbon and nitrogen sources of a compost mixture, compost selectivity or specificity is still a loosely used and not *fully* understood term. To most growers and researchers a selective compost is a substrate that is rapidly colonised by mushroom mycelium with the absence of growth of competitor 'weed' moulds. It has been assumed that a substrate with these properties is productive and yet there is little published work to substantiate this.

The work of Straatsma et al (1989), using the race tube technique first outlined by Tschierpe (1983) concluded that the presence of fungi such as *Torula thermophila*; syn. *Scytalidium thermophilum* in mushroom composts was important for the successful colonisation of the compost by the mycelium of *Agaricus* and the ultimate yield of mushrooms. These conclusions were based on the comparison of growth rate measurements of mushroom mycelium in composts that were (a) as prepared (b) autoclaved to kill resident micro-flora and (c) autoclaved substrates pre-inoculated with *S. thermophilum* and other organisms. It was found that pre-incubating autoclaved compost with 1% of untreated compost or inoculating *S. thermophilum* restored the rate of mycelial growth to the level recorded in the untreated control. Pre-incubation with the bacterium *Bacillus licheniformis* was shown to have an adverse effect on growth rate. Selectivity was therefore judged on mycelial growth rate alone. As the growth rate in autoclaved substrates was only (3-4 mm d^{-1}), it was assumed that the growth of mushroom mycelium was poor in comparison to the unautoclaved series which recorded growth between (8-9 mm d^{-1}). This assumption was based only on the macroscopic evidence that

there was no difference in mycelial density other than subtle morphological differences in mycelial stranding (shorter and curlier); an alternative interpretation of the slower growth could be that the mycelium was actually denser and that the slow mycelial advance in the autoclaved substate could be explained by the fact that the mushroom did not have to compete for nutrients with resident microflora (which were killed by the autoclaving procedure) and it dwelt in the substrate where nutrients were freely available (Thompson, 1982; Thompson & Rayner, 1982). Measurement of the extracellular enzyme laccase produced by the mushroom during vegetative culture and shown by Wood (1979) to be produced in direct proportion to fungal biomass would have been an ideal assay procedure to clarify whether the presence of thermotolerant fungi at the completion of composting actually resulted in a denser mushroom mycelium. Very recent work by Straatsma *et al* (1993), using both liquid and solid agar media, to measure growth parameters of *A. bisporus* mycelium influenced by *S. thermophilum*, shows that this organism merely affects the radial extension rate of growth rather than having a positive effect on the specific growth rate. In contrast to this work, Weigant *et al* (1992), also using *S. thermophilum* in compost, claim that the growth promoting effect that this organism has on mushroom mycelium can be explained by increased carbon dioxide production which favours increased growth of *A. bisporus* mycelium. This has been disputed in the recent work of Straatsma *et al* (1993).

As much literature had been published in recent years with regard to the importance of thermotolerant fungi and their relationship to compost selectivity, in the first series of experiments (Chapter 3) two temperatures, 45°C (favourable for thermotolerant fungi) and 55°C (more favourable for thermophilic bacteria and actinomycetes), were chosen for extending the preparation time of a commercially prepared compost. Each compost selected, was subjected to extended composting times of 1, 2 and 3 weeks at either temperature to give 6 distinct compost types with differing degrees of degradation which could then be compared with the untreated control. It became apparent from the first series of experiments that extending the

composting time at 45°C significantly improved the colonisation rate of mycelium for the majority of *Agaricus* species tested after 1 or 2 weeks of additional composting (Table 4). But, there were also significant improvements in colonisation rate for some species when grown on composts that had received prolonged composting periods at 55°C for 1 and 2 weeks (Tables 6 & 10) although further periods of composting at this temperature caused a significant decline in growth rate. In Experiment 8, the activity of the extracellular enzyme laccase was first measured after 21 days of colonisation as an assessment of fungal biomass. It was concluded that the colonisation rate as measured in 'race tubes' could not be directly related with fungal biomass accumulation as in some cases where a fast colonisation rate was recorded, laccase activity was relatively low (see Table 12; mycelial advance in composts prolonged at 45°C). In some cases,e.g. the autoclaved series of Experiment 8, the colonisation rate was visibly slower and apparently denser, and this was later confirmed by laccase assays. It must also be noted that colonisation rates of compost, in this series of experiments, were also improved by prolonging the composting phase at 55°C where thermotolerant fungi were less apparent than those prolonged at 45°C. To use mycelial colonisation rate as a measure of compost selectivity is therefore potentially misleading as it does not account for possibly the most important factor of all, fungal density. These experiments also indicate that organisms other than *S. thermophilum* may play a role in conferring selectivity on composted substrates, as both mycelial growth and fungal density were sometimes improved when composting was prolonged at 55°C.

In all experiments where composting was prolonged, there was no evidence of gaseous ammonia at the completion of composting, and pH levels were generally below 8.0. Analysis of freeze dried compost samples of Experiment 8 (H.R.I. formulation) revealed that ammonium levels (NH_4^+) fell from 0.05% compost dry matter in the control compost to 0.02% after 1 weeks composting at 45°C and remained low with further periods of composting (Figure 10). At 55°C there was also a decrease in the NH_4^+ level after 1 weeks extended composting but further periods of composting resulted

in a return to a level above 0.05% (Figure 11). It was interesting to note that there was a possible correlation between the NH_4^+ level at spawning and the rate of mycelial colonisation. In Experiment 9, where a commercial compost was used, the NH_4^+ levels remained around 0.03% of the dry matter, whether composts had received an extended composting period at 45°C or 55°C or not. Mycelial colonisation rates were also very similar in all of the composts. A possible explanation for this was that the commercial compost was much more degraded than the H.R.I. formulation, and that further periods of composting at either 45°C or 55°C had less effect at changing its microbial and chemical composition. The association of low levels of NH_4^+ and high colonisation rates were confirmed using agar plate and liquid culture studies (Chapter 3), where concentrations of NH_4^+ above 500 ppm significantly depressed mycelial growth.

The final experiment of Chapter 3 (Experiment 10) was conducted to confirm earlier findings as well as to investigate whether a rapid mycelial colonisation of a prepared substrate would ultimately lead to improved fruitbody production. Only one temperature, i.e. 45°C, was used in extending the composting time, and as shown with previous experiments, significant improvements were made in mycelial colonisation rates in composts receiving additional composting periods. Once again, in the compost taken (H.R.I. formulation), there was a significant drop in the NH_4^+ level from 0.06% to 0.017%, when composting was prolonged for a further 1 week. Ammonium levels were slightly higher in the composts that received 2 and 3 weeks additional composting, and mycelial colonisation rates in these two treatments were very similar to the compost receiving only 1 weeks additional composting. Sufficient compost volumes were produced in this experiment to carry out productivity tests and mushrooms were harvested over 12 weeks, a sufficient period to completely exhaust the substrate of nutrients. Although the control compost, which had the highest NH_4^+ level at spawning, gave the slowest colonisation rate of the 4 composts prepared, the biological efficiency (fresh weight mushrooms/100g dry matter) was the highest after 3 weeks of harvest. Even after 6 weeks of harvesting, a time regarded by most growers

as the maximum time to make most economic use of a growing facility, the control compost was shown to be as productive as the other three treatments.

The conclusions drawn from the extended composting experiments are that prolonged composting periods of commercially prepared composts at either 45°C and 55°C can significantly improve the rate of mycelial colonisation. From the results presented in this thesis, the main factor influencing the rate of mycelial growth would appear to be the NH_4^+ level at the time of spawning. A rapid colonisation rate of the compost has always been regarded as a good sign by growers, as fungal competitors do not have a chance to become established, and it is naturally assumed that a high yielding compost results. From the data presented here it is clear that additional composting, at both 45°C and 55°C, can effect a lowering of the NH_4^+ level, which can significantly improve the mycelial growth rate of the mushroom. Consequently, a quicker domination of the substrate is achieved, but it cannot be assumed that a rapidly colonised substrate will result in a high yielding substrate. Other, possibly nutritional factors, would then seem to play a role in the productivity of mushrooms from the substrate.

7.2 Humic material and its role in compost selectivity and mushroom nutrition

As all the compost samples prepared in Chapter 3 had been freeze dried and stored in air-tight bottles, it was decided to analyse the changes in humic materials as another parameter to judge compost selectivity and/or mushroom nutrition. Humic material that accumulates during the composting process (Eddy & Jacobs, 1976; Wain, 1981) and the micro-organisms embedded within it (Fermor & Wood, 1981; Sparling, Fermor & Wood, 1982; Atkey & Wood, 1983) have been shown by many researchers to be beneficial to mushroom nutrition. While it is clear that mushroom mycelium produces enzymes which degrade the micro-organisms (Fermor & Wood, 1981; Fermor, 1983; Fermor & Grant, 1985), the role of humic material remains unclear.

Eddy (1976), using microscopic examination of the dark layer that

accumulates on the straw surface of cereal straw during composting, showed that this deposit contains bacterial spores, fungal spores and hyphal fragments embedded in an amorphous matrix. This amorphous matrix was possibly derived from extra-cellular polysaccharide synthesised by bacteria and fungi during composting. After alkali extraction, he identified two fractions within this matrix which constituted 50% of the ash free compost dry matter, a carbohydrate fraction (30%) which disappeared during cropping, and a dark material termed 'humic acid' (20%) which remained throughout the vegetative and fruiting stages.

Wain (1981), also using an alkali extraction procedure, extracted the dark surface layer using a sonication method and also detected two distinct fractions, an alkali soluble/acid insoluble fraction and a debris component. The carbohydrate, protein and phenolic content of the alkali soluble/acid insoluble fraction (humic acid) was estimated to be 40%, 34% and 10% respectively, whereas the debris component constituted 40%, 12% and 4% respectively. The two fractions which collectively constituted 30% of ash-free compost dry matter (humic acid - 18%; debris fraction - 12%) was a considerable reduction on that found by Eddy (1976). Wain (1981) claimed that both fractions were utilised during the mycelial growth phase, the alkali soluble/acid insoluble fraction being reduced by as much as 75% whereas the debris component was reduced by approximately 50%.

Compost samples taken from Experiments 8 (H.R.I. formulation - short composting period; straw like; yellow/brown in colour) & 9 (Blue-Prince formulation - well degraded, dark brown/black; straw fraction not clearly discerni ble) were first chosen for analysis because these were two distinct compost types, and yet both were selective substrates for mushroom growth. Using the extraction procedure outlined in Chapter 2, two insoluble fractions were isolated from freeze dried compost samples. The alkali soluble/acid insoluble fraction component (humic fraction) of both compost types, represented about 12% of ash free compost (Figure 29) which was in a slightly lower range to the 18% found by Wain (1981). The alkali insoluble debris fraction (debris 2) separated by centrifugation from the H.R.I.

formulation, at 15% of the compost dry matter was a little higher from that found by Wain. In the well-degraded Blue Prince formulation this fraction was even higher at 30% of the compost dry matter. Collectively, these two fractions represented 27% and 45% of the total compost dry matter respectively demonstrating the wide differences that can be obtained between two compost types. The debris 2 fraction, in both compost types, was shown to increase significantly with prolonged composting times at both 45°C and 55°C (Figure 30). For the H.R.I. and Blue Prince formulations these two fractions collectively represented 42% and 62% of the total compost dry matter after 3 weeks additional composting.

Attention was first paid to the humic fraction as this fraction could be easily re-dissolved in dilute alkali simplifying further analysis. It was quite clear that a high proportion of this fraction, in excess of 35% of its dry weight was protein, soluble carbohydrates constituted around 15-20% and the phenolic content was close to 10%. Compared with Wain (1981), the protein and phenolic component estimates were very similar although the carbohydrate level at around 15-20% was much lower. In both compost types tested, the ratios of protein, carbohydrate and phenolic components remained reasonably constant within the humic fraction, irrespective of composting treatment (Figures 22 & 26). Care must be taken interpreting these results as quite large differences were recorded between control composts when analysis was performed on separate days (c.f. controls with prolonged composting series at 45°C and 55°C). A better way to present these results is to represent the estimated protein, carbohydrate and phenolic levels of the humic fraction as a proportion of ash free compost dry matter (Figures 23 & 27). It then becomes clearer that the protein fraction within the humic complex as a proportion of remaining organic material i.e. ash free compost, increases with composting time.

Analysis of the clear supernatant remaining, the fulvic fraction, was also performed. The solid material dissolved in this fraction was calculated by the difference between the starting dry weight sample and the summation of debris 1, debris 2, and humic weights. Generally the dissolved solids present

in the fulvic fraction represented approximately 20-30% of the starting compost sample dry weight (see Tables 15 to 18) of which soluble carbohydrate would appear to be a major constituent (15-20% fulvic dry weight). The protein and phenolic components were generally below 3% of the fulvic dry weight. In Figures 24 and 28,protein, carbohydrate and phenolic contents of the fulvic fraction are represented as percentages of ash-free compost. It was difficult to draw any conclusions of the effect of extended composting treatments on the carbohydrate level of the fulvic fraction, owing to the wide differences once again detected between control samples analysed on different days (Figures 24 & 28).

The analysis of the C.E.C. composts (Figure 32) gave the opportunity to study the build up of humic materials in composts with a wide range of nitrogen contents at the commencement of composting and to compare analytical data against yield of mushrooms recorded over a 6-week cropping period. No correlations could be made between the compost fractions and ultimate yield but it was observed that two composts (4 & 5) showing almost identical proportions of debris 1, debris 2, humic and fulvic fractions, produced extremely contrasting results. Although it could be argued that there were small differences in total nitrogen content at spawning i.e. 2.15 as opposed to 2.55, both composts were apparently free of ammonia at spawning and pH's were below 8.0 but compost 5 had an extremely high ammonium ion concentration (0.31% compost dry matter) in comparison to compost 4 (0.07%). This observation was consistent with our earlier findings (Expts. 8 & 10, Chapter 3), that a high NH_4^+ level inhibited mushroom growth but in this case the level was so high that mushroom mycelium was killed allowing fungal contaminants to become totally dominant.

Both the humic and the debris 2 fractions have been shown to increase with composting time, but most noticeably the latter. Both these fractions were monitored throughout the composting process in both a high and low nitrogen formulation (Tables 15, 16, 17 & 18), and the percentages of nitrogen present within each fraction determined. At the commencement of composting, debris 2 nitrogen, as a proportion of total compost nitrogen, was

2.4 and 4.4%, in the high and low formulations respectively. At the completion of composting, these levels had risen to 20.3 and 13.5% respectively. In the humic fraction there were also large increases in nitrogen during composting, rising from 10.6% to 19.8% and 9.3% to 25.8% in the high and low nitrogen formulations respectively. At the completion of composting it was noted that the summation of these two fractions, for both the high and low nitrogen formulations, amounted to a similar figure of 40% of the total nitrogen available to the mushroom.

Humic material isolated in all the experiments reported in this thesis was initially dissolved in 0.5M NaOH and then reprecipitated out of solution by pH adjustment to 2.0 with strong acid (HCl). Because humic macromolecules are highly charged, non-humic compounds, such as plant and/or microbial peptide and protein materials are normally strongly bound to them. On solubilisation and re-precipitation out of alkaline solution, it is debatable whether the reformed humic complex has the identical structure to that initially found coating the cereal straw surface. This fact must be taken into consideration when discussing estimations of the isolated humic material taken from straw surfaces and also the depletion of humic components during colonisation of the substrate by *A. bisporus*. The debris 2 fraction, on the other hand, was insoluble in dilute alkali and was easily separated by centrifugation. Another factor which one must also take into consideration is whether the milling procedure (samples were normally ball milled for 5-10 minutes to produce a fine powder) may have distorted the levels of debris 2 determined. It is possible that the debris 2 fraction at the completion of composting, as well as consisting of fungal fragments and bacterial cells, contained a certain amount of plant debris which passed through the miracloth filter paper during separation from debris 1 (the cereal straw component). Nevertheless, the procedure of extraction and estimation of humic and debris fractions from compost samples was rigidly adhered to each time and therefore the upward and downward trends identified in these fractions during composting and mycelial establishment by the mushroom was regarded as real.

7.3 Sequential degradation of compost fractions by mushroom enzymes

To gain a clearer understanding of sequential attack of mushroom mycelium on its substrate, the experiments conducted in Chapter 5, were initially concerned with vegetative culture and small scale investigations concentrated on the disappearance of debris 1, debris 2 and humic fractions. While the humic fraction appeared to be stable throughout the colonisation period (Figure 33), there did appear to be a decline in the debris 2 component which co-incided with the dramatic increase in laccase activity. Earlier work by Smith *et al* (1989), had identified distinct enzyme profiles at different depths of a colonised column of mushroom substrate that was producing mushroom fruitbodies. It was concluded that the mushroom, while totally colonising its substrate, utilises nutrients in the uppermost layers of compost first. As nutrients become depleted or exhausted in the uppermost layer, the mushroom attacks the substrate progressively in a downward manner, even though the substrate is totally colonised by mushroom mycelium. This was borne out by the endocellulase activity profiles measured throughout the depth of the substrate from the onset of fruiting and throughout a number of mushroom crops. Also reported in this paper was the fact that laccase activity measured in the lower sections of a deep layer of colonised compost, remained at a high level until the depletion of nutrients (cellulose/hemicellulose/lignin) in the upper layers. As high laccase activity was found to co-incide with the disappearance of the debris 2 fraction (See 5.1), it was therefore decided to construct 2 deep containers (1.15m high) in which further studies could continue on uncased (vegetative) and cased (productive) compost columns. As well as endocellulase and laccase activity, β-N-acetyl muramidase and β-N-acetyl glucosaminidase were also assayed within cased and uncased columns of mushroom substrate in association with the depletion of humic and debris fractions.

A further complication in measuring the declining levels of such

fractions is the fact that mushroom mycelium is steadily accumulating while plant polymers, microbial biomass and the humic material in which it is embedded is possibly decreasing. Using laccase as a measure of fungal biomass in vegetative culture (Wood, 1979), it was estimated that up to 7% of the compost dry matter could be accounted for as mushroom mycelium in well colonised composts. It is difficult to apply a correction factor to estimated levels of debris 1, debris 2, humic and fulvic fractions, but one assumes that much of the mycelium would be accounted for in the debris 1 fraction, although ball-milling freeze dried compost samples would lead to fungal fragments passing through miracloth and accumulation in the debris 2 fraction as well as release of soluble materials particularly mycelial protein into the fulvic fraction. It is also likely that cellular components of the mushroom within the freeze dried sample may dissolve in dilute alkali which then may influence the protein, carbohydrate and phenolic levels of humic acid when it is brought out of solution. All of these factors must be taken into consideration when discussing the results.

Care was taken during compost sampling from the five different layers within cased and uncased columns to select compost that was in an uninterupted mycelial pathway to the to the uppermost layer. Observations reported in earler work (Smith *et al*, 1989) were confirmed. In the cased layer, most notably the uppermost layer, laccase activity, as expected, declined at the onset of fruiting, and this co-incided with a rapid increase in endo-cellulase activity. Estimations of the debris 1 fraction clearly show a decline in this fraction (Figures 40 & 41) in association with endocellulase activity (Figure 36). In the uncased column, virtually no endocellulase activity was recorded, although laccase activity was found to remain at a high level throughout all depths of compost (Figure 37). Only very little change in debris 1 levels could be found in compost samples taken from the 5 layers of the uncased column throughout the whole experiment (Figure 40), even when corrections were made on a constant ash basis (Figure 41). The debris 2 fractions isolated from the uncased column declined with time but levels were fairly uniform throughout all the 5 layers sampled (Figures 42 & 43). In

contrast, debris 2 fractions in the cased layer declined significantly in the lower layers of compost where laccase levels remained at their highest, confirming the observations with the vegetative studies in 'race-tubes' (5.1). The two bacteriolytic enzymes, β-N-acetyl glucosaminidase and β-N-acetyl muramidase also gave different profiles in the cased and uncased column. Acetyl muramidase was clearly most active in the cased column possibly in the uppermost layers (Figure 37), although there were not such clear differences as shown with endocelluase and laccase. One interesting point to note was that the final compost samples taken from the cased column were taken after 75 days, when the casing soil had dried out discouraging fructification. The mushroom mycelium within this column had now returned to a vegetative state and this was confirmed when the the enzyme activity i.e. acetyl muramidase, was shown to be similar to that of the uncased column. It would therefore seem that this enzyme is most active when the mycelium is in a reproductive mode. Acetyl glucosaminidase activity in all 5 layers of the uncased column increased uniformly throughout the duration of the experiment whereas there were significant peaks of activity in the uppermost layers of the cased columnn, much in unison with the depletion of the debris 2 fraction (Figure 36). If it can be assumed that mushroom biomass within the cased and uncased columns was identical at the time of casing, and little change was to occur during the growth and reproductive phases, the distribution of mushroom dry weight material throughout the extracted fractions, at whatever stage of sampling, would be similar and therefore the changes identified in the humic and debris 2 fractions are real changes.

Another factor to consider is that it is highly likely that humic material in its natural condition has fixed amounts of e.g. protein macro-molecules, as part of its complex, which become utilised by the mushroom during the growth phases. On extraction of the humic material from the cereal straw surfaces after a number of mushroom flushes, one would expect to see a much more rapid decline in humic protein than that reported in Chapter 5. As a rapid decline in humic protein was not found, two hypotheses could be put forward (a) nitrogen as protein within the humic fraction is not mobilised by

Agaricus to any great extent or (b) the humic material extracted by sonication in dilute alkali and followed by reprecipitation in strong acid may be structurally different to the raw material deposited on the cereal straw surfaces prior to alkali extraction. It is well recognised that the study of nitrogen in humic substances is complicated by the presence of co-extracted non-humic nitrogen containing compounds (Anderson *et al*, 1989). A possible explanation therefore for the apparent stable levels of humic material detected throughout the growth phase could be that the humic complex changes during solubilisation in alkali, and that closely bonded macromolecules are released. On re-precipitation from the supernatant solution, the available bonding sites are once again filled up with non-humic compounds such as plant and/or microbial peptide and protein materials giving the impression that no major structural changes have occurred during mycelial establishment of the substrate.

7.4 Concluding Comments

As a result of these investigations, a further advance in the knowledge of factors that confer selectivity to a composted substrate favouring *Agaricus bisporus* colonisation has been made. Compost selectivity is only achieved when three sets of conditions are fulfilled, each set of conditions being a natural progression of the one before. It has been an established fact since the early 1930's that to produce a selective medium on which the mushroom grows, it is necessary to reduce the soluble carbon and nitrogen forms of an organic substrate (compost) to a low level as the mushroom is generally outcompeted by both bacterial and fungal contaminants if these readily available sources are present on inoculation. This can be regarded as the first set of conditions that is so essential in encouraging mycelial growth. Another well established fact is that mushroom mycelium is inhibited by the presence of ammonia and that at very high concentrations, no mycelial growth will occur. It has become standard practice to complete phase 2 composting only when gaseous ammonia levels in the compost are below 10 ppm (Draeger) and a red litmus paper no longer turns

blue i.e. the compost pH is no higher than 8.0 (Gerrits, 1988). While these factors have been regarded as reliable indicators of compost selectivity and of a good spawn run to follow, there are occasions when mycelial establishment is poor even though all the indicators would suggest otherwise. From the 'race tube' experiments carried in these investigations it is quite clear that the ammonium level at the completion of composting may be a more important indicator of compost selectivity than the measurement of gaseous ammonia. Normally, at the completion of Phase 2, the compost is free of gaseous ammonia, while the ammonium content of the compost as a percentage of the dry matter falls to 0.1% or lower. Although the mushroom can tolerate such low levels and possibly utilise it as a source of nitrogen (Gerrits, 1988), mycelial growth rate can be affected. During the analysis of the 6 C.E.C. composts (Chapter 4), it was clearly demonstrated, that there were occasions (composts 5 & 6) when composts were found clear of gaseous ammonia, the pH was below 8.0, and yet ammonium levels were in excess of 0.3%.(see Figure 32). Composts within this category normally failed to give a crop due to poor mycelial establishment. While the reduction of gaseous ammonia in the compost to below 10 ppm at spawning is one factor which ensures a quick mycelial establishment, the reduction of the ammonium level to below 0.1% of the dry matter can also be regarded as the second factor which can have a major influence on mushroom growth. While it must be emphasised that it is extremely important for the mushroom to colonise its substrate as quickly as possible, to out-compete fungal competitors, these studies have also shown that a rapid colonisation of the substrate does not necessarily guarantee that a high yield of mushrooms is to follow.

Finally, the conversion of soluble nitrogen into insoluble forms such as microbial protein (as shown in the debris 2 fraction) and humic bound protein can be regarded as the third set of conditions influencing compost selectivity and possibly more importantly, compost productivity. These important nitrogen forms represent approximately 40% of the total nitrogen available to the mushroom at the time of spawning. While it is easy to assess the compost fractions at the completion of composting, assessing their depletion

with time becomes an extremely difficult task as mushroom biomass within the substrate can represent more than 7% of the total compost dry matter. No attempt has been made within this thesis to modify the gathered data during the growth phase for mushroom mycelium interference but there are clear indications from this work that the humic bound protein and the microbial protein of debris 2 are important nitrogen sources for the mushroom.

While it has been convenient to carry out an intensive study using freeze dried compost samples, as many compost types (or treatments) can be evaluated on the same day, it is suggested that further work should concentrate on the extraction of fresh weight compost samples. Although a less convenient approach than using freeze dried samples (as larger quantities of compost will be required to reduce sampling error and the moisture content will have to be accu rately determined to calculate compost sample dry weight), it is likely, for the reasons outlined earlier, that a more accurate assessment of humic and debris 2 fraction utilisation by the mushroom would result. Further work at evaluating humic and debris 2 fractions (and components within) from a wide range of compost types is suggested in order to identify nutritional indices that relate to mushroom yield.

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APPENDIX 1 LIQUID MEDIA (based on Treschow, 1944)

Six stock solutions were sterilised separately, A-F being autoclaved at 121°C at 15 p.s.i. Solution F was filter sterilised using a pre-autoclaved Millipore filter (0.22 μm). The composition of the stock solutions were as follows:-

A.	KCl	2g
	$MgSO_4.7H_20$	2g
	Mineral solution (See below)	10ml
	Water to:-	**100ml**
B.	$CaCl_2.6H_20$	4g
	Water to:-	**100ml**
C.	$FeCl_3.6H_20$	200mg
	Water to:-	**100ml**
D.	KH_2PO_4	2.61g
	Na_2HPO_4	114mg
	Water to:-	**500ml**
E.	D-glucose	100g
	Water to:-	**400ml**
F.	L-glutamate (monosodium salt)	8.5g
	Vitamin Stock (See below)	10ml
	Water to:-	**400ml**

The mineral and stock solutions were composed of the following:-

MINERAL	$CuSO_4.5H_2O$	26mg
	$MnCl_2.4H_2O$	200mg
	$Na_2MoO_4.2H_2O$	56mg
	H_3BO_3	30mg
	$ZnSO_4.7H_2O$	20mg
	$CoCl_2.6H_2O$	70mg
	Water to:-	1l
VITAMIN	Thiamine hydrochloride	20mg
	Biotin	2mg
	Water to:-	100ml

The final medium was made up of 10ml each of A, B and C, plus 500ml of D, 40ml of E, 40ml of F and sterile water to to 1l.

APPENDIX 2. ANALYTICAL DATA FOR EXPERIMENT 8 COMPOSTS
(H.R.I. Formulation)

4 replicate compost samples (0.3g freeze dried and milled) taken for extraction with 0.5N NaOH

Rep. No. Treatment	Debris 1	Debris 2	Humic (ash free)	Fulvic
Control	0.1768	0.0427	*	*
	0.1778	0.0482	*	*
	0.1710	0.0535	0.0321	0.0434
	0.1756	0.0435	0.0313	0.0496
+ 1 week (45°C)	0.1471	0.0618	*	*
	0.1359	0.0800	*	*
	0.1488	0.0726	0.0321	0.0465
	0.1502	0.0723	0.0318	0.0457
+ 2 weeks (45°C)	0.1536	0.0728	*	*
	0.1294	0.0831	*	*
	0.1606	0.0720	0.0258	0.0416
	0.1342	0.0832	0.0286	0.0540
+ 3 weeks (45°C)	0.1053	0.1066	*	*
	0.1162	0.1041	*	*
	0.1177	0.1014	0.0338	0.0471
	0.1292	0.1037	0.0342	0.0329

* Two replicates kept for further analysis

Fulvic fraction solids calculated by summation of debris 1, debris 2, and ash-free humic fractions and difference between 0.3g starting sample

APPENDIX 3. ANALYTICAL DATA FOR EXPERIMENT 8 COMPOSTS
(H.R.I. Formulation)

4 replicate compost samples (0.3g freeze dried and milled) taken for extraction with 0.5N NaOH

Rep. No. Treatment	Debris 1	Debris 2	Humic (ash free)	Fulvic
Control	0.1803	0.0435	*	*
	0.1822	0.0424	*	*
	0.1723	0.0429	0.0301	0.0547
	0.1785	0.0474	0.0305	0.0564
+ 1 week (55°C)	0.1310	0.0770	*	*
	0.1576	0.0591	*	*
	0.1345	0.0685	0.0347	0.0623
	0.1318	0.0739	0.0371	0.0572
+ 2 weeks (55°C)	0.1077	0.1010	*	*
	0.1201	0.0870	*	*
	0.1199	0.0951	0.0356	0.0494
	0.1166	0.0985	0.0350	0.0499
+ 3 weeks (55°C)	0.1262	0.0951	*	*
	0.1251	0.0907	*	*
	0.1444	0.0843	0.0325	0.0388
	0.1283	0.0869	0.0355	0.0493

* Two replicates kept for further analysis

Fulvic acid solids calculated by summation of debris 1, debris 2 and ash-free humic fractions and difference between 0.3g starting sample

APPENDIX 4. PROTEIN / CARBOHYDRATE / PHENOLIC content (%) of ASH-FREE HUMIC FRACTION of Experiment 8 composts (H.R.I.)

Composts prolonged at 45°C

Treatment	Control	+ 1 week	+ 2 weeks	+ 3 weeks
Protein	32.99	50.01	50.58	49.26
	46.00	49.05	46.67	46.05
Carbohydrate	14.39	14.48	13.25	16.47
	14.12	12.57	13.70	21.05
Phenolic	10.93	11.63	11.95	10.94
	11.13	11.35	11.31	10.54

Composts prolonged at 55°C

Treatment	Control	+ 1 week	+ 2 weeks	+ 3 weeks
Protein	37.13	37.34	37.07	43.78
	35.90	31.29	39.85	40.98
Carbohydrate	22.24	22.19	20.92	20.99
	18.26	16.30	22.28	19.97
Phenolic	9.20	9.61	9.04	10.38
	9.01	8.09	9.64	9.85

APPENDIX 5. PROTEIN / CARBOHYDRATE / PHENOLIC CONTENT (%) of FULVIC ACID FRACTION of Experiment 8 composts (H.R.I.)

Composts prolonged at 45°C

Treatment	Control	+ 1 week	+ 2 weeks	+ 3 weeks
Protein	3.63	1.93	2.04	1.87
	3.17	2.10	1.61	3.06
Carbohydrate	24.35	23.33	17.06	20.38
	21.67	19.36	13.51	24.24
Phenolic	3.45	2.83	2.57	2.54
	3.02	2.88	2.16	3.49

Composts prolonged at 55°C

Treatment	Control	+ 1 week	+ 2 weeks	+ 3 weeks
Protein	0.31	0.61	0.61	0.69
	0.34	0.36	0.60	0.37
Carbohydrate	15.17	13.35	18.42	28.86
	12.67	15.13	19.63	26.77
Phenolic	1.95	1.73	2.06	2.75
	1.86	1.74	2.00	2.06

APPENDIX 6. ANALYTICAL DATA FOR EXPERIMENT 9 COMPOSTS
(Blue Prince Formulation)

4 replicate compost samples (0.3g freeze dried and milled) taken for extraction with 0.5N NaOH

Rep. No. Treatment	Debris 1	Debris 2	Humic (ash free)	Fulvic
Control	0.1332	0.0936	*	*
	0.1366	0.1005	*	*
	0.1413	0.0951	0.0311	0.0411
	0.1426	0.0980	0.0288	0.0479
+ 1 week (45°C)	0.1253	0.1008	*	*
	0.1131	0.1034	*	*
	0.1246	0.1005	0.0365	0.0384
	0.1325	0.0979	0.0344	0.0352
+ 2 weeks (45°C)	0.1146	0.1236	*	*
	0.1138	0.1134	*	*
	0.1127	0.1185	0.0317	0.0371
	0.1101	0.1213	0.0385	0.0301
+ 3 weeks (45°C)	0.0934	0.1348	*	*
	0.1056	0.1270	*	*
	0.0787	0.1431	0.0377	0.0405
	0.0832	0.1413	0.0336	0.0419

* Two replicates kept for further analysis

Fulvic acid solids calculated by summation of debris 1, debris 2 and ash-free humic fractions and difference between 0.3g starting sample.

APPENDIX 7. ANALYTICAL DATA FOR EXPERIMENT 9 COMPOSTS
(Blue Prince Formulation)

4 replicate compost samples (0.3g freeze dried and milled) taken for extraction with 0.5N NaOH

Rep. No. Treatment	Debris 1	Debris 2	Humic (ash free)	Fulvic
Control	0.1539	0.0851	*	*
	0.1619	0.0840	*	*
	0.1551	0.0910	0.0317	0.0222
	0.1416	0.1024	0.0279	0.0281
+ 1 week (55°C)	0.1287	0.1038	*	*
	0.1145	0.1051	*	*
	0.1384	0.0967	0.0334	0.0315
	0.1318	0.1050	0.0341	0.0291
+ 2 weeks (55°C)	0.1097	0.1161	*	*
	0.1146	0.1127	*	*
	0.0970	0.1201	0.0372	0.0457
	0.1047	0.1193	0.0336	0.0424
+ 3 weeks (55°C)	0.0927	0.1379	*	*
	0.1020	0.1241	*	*
	0.0975	0.1263	0.0332	0.0430
	0.0942	0.1285	0.0336	0.0437

Two replicates kept for further analysis

Fulvic acid solids calculated by summation of debris 1, debris 2 and ash-free humic fractions and difference between 0.3g starting sample.

APPENDIX 8. PROTEIN / CARBOHYDRATE / PHENOLIC content (%) of ASH-FREE HUMIC FRACTION of Experiment 9 composts (BLUE PRINCE)

Composts prolonged at 45°C

Treatment	Control	+ 1 week	+ 2 weeks	+ 3 weeks
Protein	39.54	40.68	46.37	34.21
	40.83	50.58	38.18	43.03
Carbohydrate	18.32	17.94	20.97	16.25
	18.29	22.87	16.23	18.36
Phenolic	8.47	8.31	10.17	7.55
	9.46	10.65	8.11	8.48

Composts prolonged at 55°C

Treatment	Control	+ 1 week	+ 2 weeks	+ 3 weeks
Protein	40.22	39.07	39.11	43.82
	52.15	43.72	40.62	40.62
Carbohydrate	19.65	18.65	18.14	19.12
	22.75	20.14	19.28	16.96
Phenolic	8.54	7.51	7.12	7.63
	10.03	8.16	7.58	7.14

APPENDIX 9. AMMONIUM ION CONCENTRATIONS (% dry compost matter) present in Compost Expts. 8, 9 & 10.

Experiment 8.

Composting temperature	Control	+ 1 week	+ 2 weeks	+ 3 weeks	Lsd (5%)
45°C	0.052	0.019	0.024	0.025	0.004
55°C		0.022	0.032	0.054	

Experiment 9. (Blue Prince)

Composting temperature	Control	+ 1 week	+ 2 weeks	+ 3 weeks	Lsd (5%)
45°C	0.029	0.027	0.024	0.030	0.001
55°C		0.035	0.032	0.021	

Experiment 10.

Composting temperature	Control	+ 1 week	+ 2 weeks	+ 3 weeks	Lsd (5%)
45°C	0.061	0.017	0.022	0.026	0.015

APPENDIX 10. PROTEIN / CARBOHYDRATE / PHENOLIC CONTENT (%) of FULVIC ACID FRACTION of Experiment 9 composts (BLUE PRINCE)

Composts prolonged at 45°C

Treatment	Control	+ 1 week	+ 2 weeks	+ 3 weeks
Protein	1.70	1.64	1.81	1.06
	1.38	1.73	1.69	1.21
Carbohydrate	15.80	18.22	17.60	18.88
	11.94	27.18	22.69	18.78
Phenolic	2.23	2.26	2.34	1.97
	1.81	3.03	2.65	1.90

Composts prolonged at 55°C

Treatment	Control	+ 1 week	+ 2 weeks	+ 3 weeks
Protein	2.97	1.33	1.20	0.97
	1.20	1.58	1.08	0.96
Carbohydrate	42.56	29.11	20.67	17.83
	34.05	31.68	21.98	16.97
Phenolic	4.95	2.76	1.90	1.79
	3.80	2.98	2.05	1.76

APPENDIX 11. YIELD of MUSHROOMS (g) and PRODUCTION EFFICIENCIES (P.E.) obtained from 4 compost types (Experiment 10).

Compost treatment	Control	+1 week	+2 weeks	+3 weeks
Av. wt/pot/1 week	10.23	10.50	8.80	9.39
P.E.	**33.93**	**35.81**	**31.65**	**33.26**
Av. wt/pot/3 weeks	16.22	12.91	10.15	9.39
P.E.	**53.79**	**44.03**	**36.51**	**33.26**
Av.wt/pot/6 weeks	18.17	15.58	16.31	11.41
P.E.	**60.26**	**53.13**	**58.66**	**40.41**
Av.wt/pot/9 weeks	23.10	23.71	26.58	18.87
P.E.	**78.27**	**80.86**	**95.61**	**66.84**
Av.wt/pot/12 weeks	34.56	29.95	32.90	29.28
P.E.	**114.62**	**102.14**	**118.34**	**103.71**

APPENDIX 12 ESTIMATION of MUSHROOM BIOMASS during colonisation of compost in Race Tubes (See 5:1)

Sample time	Laccase assay		Wt.of mushroom mycelium (g/20g fr.wt. compost sample)	mushroom mycelium (% compost dry matter)
	μmol O_2 min^{-1} /ml sample	μmol O_2 min^{-1} /160ml		
At spawning	*	*	*	*
+ 10 days	0.021	3.39	0.033	0.48
+ 17 days	0.153	24.48	0.239	3.44
+ 26 days	0.331	52.94	0.519	6.76
+ 41 days	0.353	56.52	0.554	7.05

* No sample taken at the time of compost ino

APPENDIX 13. ASH CONTENTS of DEBRIS 1 and DEBRIS 2 FRACTIONS (See 5:1)[* Average weight taken of two 0.3g extracted compost samples].

Sampling time	At fill	At sp.	+10 days	+17 days	+26 days	+41 days
Debris 1	0.2010*	0.1623	0.1467	0.1424	0.1560	0.1448
Ash %	9.98	7.91	6.95	6.26	8.75	10.58
AF Debris 1	0.1809	0.1494	0.1365	0.1334	0.1423	0.1302
Debris 2	0.0772*	0.0640	0.0700	0.0730	0.0542	0.0557
Ash %	32.95	35.04	35.78	36.32	36.33	38.63
AF Debris 2	0.0115	0.0415	0.0415	0.0464	0.0345	0.0341

Appendix 14. Moisture content of 5 separate compost layers within the cased and uncased columns